THE NUTRITIONAL SIGNIFICANCE OF INOSITOL

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During the past few years interest in the nutritional properties of inositol has increased to such an extent that a review of published work in this field has seemed advisable, not only to survey and evaluate what has already been done, but also to delineate some of the paths that future investigators may find profitable. Limitations of space will not permit an exhaustive review of the subject of inositol, and hence the rôle which this substance plays in intermediary metabolism as well as some physiological and chemical considerations of interest must be passed over. An attempt will be made, however, to summarize briefly present knowledge of the requirements of various species for inositol in the diet.

INOSITOL AS A MICROBIAL GROWTH FACTOR

The earliest observation on the nutritional significance of inositol was that of Eastcott (1) who isolated a growth stimulant for yeast from tea, and showed that it was meso-inositol. Eastcott's observation did not at first find ready acceptance (2), possibly due to inadequacies in biotin and pantothenic acid (2, 3) of some of the basal media used, but now has been amply confirmed and is used as the basis of the quantitative method for the estimation of inositol (4).

Several other fungi have been shown to require inositol for maximal growth (5, 6, 7), but thus far it has not been demonstrated to be required by any bacterial species. Indeed, many bacteria do not contain the substance in detectable amounts (8, 9), although certain others, notably Pseudomonas and several sporeforming species, contain considerable quantities. If this failure to detect inositol in several bacteria really is due to its absence from these forms and does not merely represent lack of sufficient sensitivity of the assay procedure, it constitutes an exception to the theory now current that all those water-soluble vitamins which are not required in the medium are synthesized by the cell.

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INOSITOL AS A DIETARY ESSENTIAL FOR ANIMALS

Mice

The present interest in the nutritional significance of inositol stems from the work of Woolley who in 1940 (10, 11) showed that a disease of mice of dietary origin, characterized by marked alopecia and retarded growth, was cured by inositol. A striking sign of the disease was the pattern of the alopecia (12). Hair was never lost from the head or tail or from the legs below the knees, while the areas of loss of hair on most other parts of the body were bilaterally symmetrical and in these areas the alopecia was nearly complete. In contrast to these findings Martin (13) observed only slight alopecia in mice fed a diet deficient in inositol. Woolley (9, 14) observed that the incidence of signs of inositol deficiency was usually not above 50% and that the amount of pantothenic acid in the diet had a marked influence on the appearance of the condition. Thus, in the absence of pantothenic acid, alopecia developed even though the ration contained adequate inositol. However, in the presence of large amounts of pantothenic acid and in the absence of inositol some animals still developed signs of inositol deficiency, and died unless the latter compound was administered. Analyses of the mice for inositol showed that the level fell to about half the normal value in the carcasses of affected animals (9).

While not of importance nutritionally, the work of Laszlo and Leuchtenberger (15) is of interest. These investigators reported that the daily intravenous injection of minute amounts of inositol prevented the development of transplanted tumors in mice. Strangely enough the effect was only observed following intravenous administration and was not seen when other routes were employed.

Rats

Shortly after the demonstration of the effect of inositol on alopecia in mice the influence of this substance on various experimental diseases of rats was examined. Gavin et al. (16, 17) reported that a type of fatty liver of rats induced by the feeding of biotin concentrates was treated successfully with inositol. Since that time several other investigators (18, 19) have observed beneficial effects of inositol on fatty livers in rats even when the fatty livers were produced without the aid of biotin. A lipotropic action of inositol in man under certain conditions has been reported (20). Pavcek and Baum (21) stated that the alopecia around the eyes seen in certain deficient animals (the so-called "spectacle eye") was promptly cured with inositol. Subsequently Nielsen and Elvehjem

(22) were inclined to view "spectacle eye" as a specific sign of biotin deficiency not curable by inositol.

There are data which indicate that the rate of growth of rats fed an otherwise adequate, highly purified diet is increased by inositol (18). On the other hand, rats have been raised to maturity on a diet free of inositol (23, 24). Martin (25) reported that the addition of p-aminobenzoic acid to a highly purified ration produced a syndrome characterized by poor growth and changes in fur which was preventable by inositol. Somewhat analogous effects have been observed by others (26, 27). Curiously enough, the diet when supplemented with neither p-aminobenzoic acid nor inositol was adequate for the rats. Furthermore when this ration was supplemented with inositol alone a definite deleterious effect was observed which was prevented by p-aminobenzoic acid.

Sure (28) presented data which indicated that inositol and p-aminobenzoic acid, especially the former, when fed to mothers receiving a highly purified diet, considerably improved the percentage survival of newborn rats. In a subsequent paper (27) Sure attributed the action largely to p-aminobenzoic acid. Climenko and McChesney (29), on the other hand, attributed the action largely to inositol.

When diets of more complex nature than the ones in the above studies were used, a striking, beneficial effect of inositol on rats was observed by various workers. Thus Cunha et al. (30) found that when rats were fed a diet made up largely of corn, soybean meal, salts, and alfalfa, they grew suboptimally and developed alopecia, similar in distribution and character to that observed by Woolley in inositol-deficient mice. That the syndrome represented inositol deficiency in rats was readily demonstrated by feeding this compound. Shortly after the appearance of this paper Nielsen and Black (31) showed that when rats were fed a highly purified diet containing all of the known vitamins including biotin and folic acid, and sulfasuccidine, they failed to grow optimally and developed alopecia. Growth was restored and alopecia prevented by administration of inositol. The sulfonamide had in some way brought out a need for inositol.

Cotton rats

McIntire et al. (32) reported that the addition of inositol to a highly purified diet practically doubled the rate of growth of cotton rats. This is the greatest effect on growth rate which has been observed with any species in which highly purified diets supplied with ample amounts of the known vitamins (and not supplemented with sulfonamides) were used.

Guinea pigs

Hogan and Hamilton (33) observed that the rate of growth of guinea pigs fed on a partially purified diet was increased by the addition of inositol.

Hamsters

Hamilton and Hogan (34) reported that, while the rate of growth of hamsters was not increased by inositol, those animals raised on the inositol-deficient diet had difficulty in reproduction. Many of the young were born dead or as bloody shapeless masses, and some of the mothers succumbed during parturition. Animals fed inositol did not show these signs. Cooperman et al. (35) observed increased rate of growth in hamsters following addition of inositol to a purified diet.

Chickens

Hegsted et al. (36) reported that the rate of growth of chicks fed a partially purified diet was increased slightly by addition of inositol. Dam (37) observed that inositol added to a tocopherol-deficient diet prevented encephalomalacia and exudative diathesis — two signs frequently seen in tocopherol-deficient chicks. In this connection it is interesting to speculate whether the substance in soybean oil reported to be necessary in addition to tocopherol for the cure of tocopherol deficiency in chicks (38), may not be lipositol (39) the inositol-containing phosphatide which is present in large amounts in soybeans.[1]

SYNTHESIS OF INOSITOL BY ANIMALS

In considering the nutritional significance of inositol it is necessary to note that several species of animals have been shown to synthesize the compound. Vohl in 1858 (40) was able to isolate quantities of inositol from the urine of a man with diabetes insipidus far in excess of the amount we now know could have been obtained from the food. The extra inositol excreted probably arose from synthesis, or possibly from tissue breakdown. Following this lead, Needham in 1924 (41) showed that rats rendered polyuric by administration of salt excreted more inositol than was ingested. Since the excretion of this excess of inositol continued over long periods, it was concluded that the rats were synthesizing the substance. Woolley (9) demonstrated that mice fed a synthetic diet frequently synthesized inositol in amounts almost equal to the minimal effective dose of the compound for this species. At least one point of origin of this synthesized inositol was shown to be the in-

[1] Patrick has recently (Gibson Island Conference, 1944) recognized that this effect is merely due to the antioxidant action of various phosphatides.

testinal flora of the mice. Needham (42) and Snell and Quarles (43) reported that during the incubation of eggs considerable inositol was synthesized. However, Woolley (44) showed that these observations on eggs were in error due to inadequacies of the methods of analysis used, and that what actually occurred during incubation was the conversion of an inositol compound into free inositol.

IS INOSITOL A DIETARY ESSENTIAL?

It is quite evident that there is no unanimity of opinion as to whether or not inositol should be regarded as a dietary essential. The fact that several workers have been able to obtain good growth of rats, mice, and chicks when these animals were fed highly purified diets very low in or devoid of inositol, and the fact that some of these species have been shown to synthesize inositol, must be pitted against the fact that several groups of workers have been able to observe moderate or marked beneficial results from the addition of inositol to the diets of mice, rats, guinea pigs, hamsters, cotton rats, and chicks. Several, but not all, of the less well marked responses and all of the failures have been observed in animals fed highly purified diets wherein it is customary to include amounts of the pure, water-soluble vitamins considerably in excess of the levels of these which occur in natural diets. Furthermore, there may be substances in natural foods which, although not themselves necessary to the species under investigation, nevertheless exert a marked influence on the need for exogenous inositol. Thus, Gavin and McHenry (16) showed that the addition of biotin to purified diets for rats caused the production of a disease (fatty livers) which was due to a dietary deficiency of inositol. Woolley (9, 14) showed that the incidence of alopecia as a sign of inositol deficiency in mice was in some measure dependent on the amount of pantothenic acid fed. In this connection, too, it is of interest to recall that Cunha et al. (30) observed their clear-cut case of inositol deficiency in rats not on a highly purified diet, but rather on one compounded from natural foodstuffs. Finally, in connection with the question of synthesis of inositol, it must be recalled that rats are able to synthesize choline (45) even though it can be demonstrated that they require choline or other suitable source of labile methyl groups in the diet.

The production of inositol deficiency in rats fed a highly purified diet plus sulfonamide drugs (31) indicated either that these drugs increased the demands of the organism beyond its synthetic capacities or that the intestinal microorganisms which were checked by the drugs were acting as a source of inositol. The demonstration in mice that in-

ositol-synthesizing microorganisms were present in the intestinal tracts of animals which did not exhibit signs of deficiency, or of those which cured spontaneously, but were not detectable in animals with signs of the deficiency (9) may lend support to the hypothesis that failure to observe inositol deficiency is due to the presence in the intestinal tract of bacteria which synthesize the compound. However, much more work will have to be done before this postulate can be regarded as established.

There are, finally, the several instances in which different groups of investigators have noted rather marked beneficial effects of inositol on several species of animals fed on highly purified diets. In some of these cases death resulted unless inositol was present in the diet. These observations were referred to in the preceding sections.

METHODS OF ANALYSIS

The most satisfactory method for the analysis of inositol now available is that which depends on the fact that inositol is a stimulant of the growth of Saccharomyces cerevisiae (4). It is specific, rapid, relatively accurate, and allows the separate estimation of both free and total inositol (9). Furthermore, it has the very considerable advantage that since it requires only minute amounts of inositol, very small samples of tissue suffice for a determination. Williams et al. (46) proposed a somewhat similar method, but it suffers from the fact that enzymic hydrolysis of the sample is employed to free combined inositol. One of the forms in which inositol occurs in tissues, namely lipositol (39), is not readily attacked by enzymes.

Several chemical methods have been advocated for the estimation of inositol. The combustion method of Needham (47) lacks specificity and does not determine some of the combined forms. Young (48) proposed a method based on the reaction of inositol with potassium iodomercurate, but the procedure is lengthy, requires large samples of tissue, and may lack specificity since many polyhydroxy compounds react with the reagent. Platt and Ghock (49) have advocated a method based on the fact that inositol, like other glycols, reacts with periodate. Steps were introduced to eliminate other naturally occurring glycols from the tissue extract before analysis. The results on comparable animal tissues were in reasonable agreement with those reported from the use of the yeast-growth method.

STRUCTURAL SPECIFICITY OF INOSITOL ACTION

The only compound which has yet been found to have appreciable inositol action for the growth of Saccharomyces cerevisiae is meso-

inositol (3). Some slight activity was shown by mytilitol, a methyl inositol obtained from mussels (50), and for inositol monophosphate. d-Inositol, l-inositol, the monomethyl ethers of these compounds, i. e., pinitol and quebrachitol, quercitol, quinic acid, inositol hexaacetate, phytin, inositol tetraphosphate, soybean lipositol, and inosose were inactive for the yeast. In mice, the results were similar except that the esters of inositol, namely, phytin, inositol hexaacetate, and soybean lipositol were active. These facts are important because most of the above compounds occur naturally (see below). It is therefore necessary to know which of them are of importance nutritionally. Also, since the esters of inositol are active for the animal but not for yeast, it is essential to convert these esters to the free compound before proceeding to measure the inositol content of foods by the yeast-growth method if a true evaluation of the foods is to be had.

It must be inferred from the results of Cunha et al. (30) that the inositol of phytin is not available to the rat. The ration which they used must have contained considerable phytin. If this inference is correct, the rat differs from the mouse in this respect.

CHEMICAL STRUCTURE OF INOSITOL

For a substance which has been under investigation for as long as inositol, it is rather surprising to realize that its complete chemical structure has been established only within the last 2 years. The reader will infer that there were special problems involved in order that this should be so. It is beyond the scope of this review to go into the steps by which the structure was finally established, but reference must be made to the work of Posternak (51) and of Dangschat and Fischer (52) who showed that the 6 hydroxyl groups are arranged about the cyclohexane ring in such a manner that four are below the plane of the ring and two are above. The two hydroxyls that are above the ring are in 1, 3 position.

NATURAL OCCURRENCE OF INOSITOL AND ITS DERIVATIVES

A number of isomers and analogs of inositol occur naturally, but since they do not exhibit inositol action nutritionally, only their existence will be noted here, and the reader will be referred to various handbooks such as that of Klein (53) for more extensive information.

Meso-inositol itself occurs in nature in at least four forms: free inositol, phytin, lipositol, and a water soluble, non-dialyzable complex. Free inositol has been isolated from both plant and animal sources. Phytin, the calcium and magnesium salt of inositol hexaphosphate, was

thought of until recently as an exclusive plant constituent. Its presence in many seeds has been known for decades. However, Rapoport (54) has shown that the erythrocytes of species such as the chicken and turtle in which these cells are nucleated contain appreciable quantities of phytin.

Inositol as a constituent of special phosphatides has been known from the work of Anderson (55) who isolated it from the phosphatides of the tubercle bacillus, and from the work of Klenk and Sakai (56) who obtained inositol monophosphate from soybean phosphatides. The widespread occurrence of inositol in phosphatides, however, was seen when Folch and Woolley (57) showed that it was combined in the cephalin fraction of brain and spinal cord and thus recognized a new inositol-containing phosphatide in animal tissues. Although the pure phosphatide has not yet been obtained from animal sources, a similar compound has been isolated from soybeans (39) and shown to be composed of inositol monophosphate in glycosidic linkage with galactose, and combined with ethanolamine, tartaric acid, oleic acid, and saturated fatty acids. It was named lipositol.

The occurrence of inositol as an integral part of a phosphatide and the rôle of inositol in the prevention of fatty livers bring to mind the occurrence of choline in a phosphatide and its vitamin-like action in prevention of fatty livers of a different character. Perhaps the formation of lipositol is one of the uses to which dietary inositol is put by the animal.

The existence of an inositol complex in heart muscle has seemed probable from the early observations of Rosenberger (58), but it was not until Winter's work (59) that this complex was demonstrated by controlled experiments. During autolysis of heart muscle of certain species the complex was broken down to free inositol. It does not seem probable that this complex in heart muscle is lipositol since that organ is low in this compound, and since lipositol is not readily hydrolyzed during autolysis of tissues. Woolley (11) recognized a water-soluble, non-dialyzable inositol complex in liver, and showed (9) that inositol complexes were present in most of the tissues that were examined.

Most plant and animal tissues thus far examined have been found to contain between 0.05 and 0.5% of total inositol on a dry weight basis (4, 60). Heart was the richest natural source since 1.6% of the dry weight of this organ was found as inositol. In the few tissues which have been analyzed, approximately one-half of the total inositol was free (9), although the proportion which was uncombined varied from tissue

to tissue. The amount of total inositol in many natural diets is of the same order of magnitude as that which has been found to prevent signs of inositol deficiency in the various species examined.

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THE EFFECTS OF SUGARS ON THE RESPIRATORY EXCHANGE OF CATS [1]

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In our general program of research on the variations in the respiratory quotient and in the total metabolism of different species of animals after the ingestion of sugars, the cat has been studied as a representative of the Carnivora. Usually the diet of the cat is mainly of animal origin, although domesticated cats can be trained to eat foods of plant origin.

METHODS AND PROCEDURE

The respiratory exchange of five nearly adult or fully grown cats was determined by the open-circuit method, proportionate samples of the ventilating air current being collected in the Fox bag (Carpenter and Fox, '31a) and subsequently analyzed by means of a gas-analysis apparatus (Carpenter, '33). The gas-analysis apparatus was standardized daily by analyses of samples either of outdoor air or of air in which ethyl alcohol was burning. The average respiratory quotient of 129 samples of air passing over burning alcohol was 0.665 with a standard deviation of $\pm$ 0.0059.

The respiration experiments [2] were made from 16 to 24 hours after food had been removed from the cats. The room temperature at which the cats were kept prior to the experiments varied according to the season, but the measurements of respiratory exchange were made at environmental temperatures between 20° and 28°C., usually 22° to 26°C. Observations were made during eight successive ½-hour periods under basal conditions (no dose), after ingestion of 75 ml. of water as a control, and after ingestion of 10 gm. of glucose, fructose, galactose, lactose, maltose, or sucrose, and combinations of 5 gm. each of glucose and galactose or of glucose and fructose. The sugars were dissolved in 50 ml. of water at 37°C. and given by stomach tube, and 25

[1] A preliminary report has been presented in the Federation Proceedings, vol. 3, p. 93 ('44).

[2] The respiration experiments were carried out with the technical assistance of Mr. Basil James.

ml. of water was used to rinse the funnel and the tube. The measurements of the respiratory exchange were usually begun about 20 minutes after the feeding.

All the cats were male except A. Cat G was old, not in good physical condition, and finally died of some pulmonary difficulty.

In the comparisons made in the following pages between cats and man, the statements regarding man are based on reports previously published by Carpenter and Fox ('30a, '30b, '31b, '31c), Carpenter and Lee ('32a, '32b, '37a, '37b, '37c, '38), and Root and Carpenter ('42).

RESULTS

Respiratory quotient (R. Q.)

The average respiratory quotients in the successive ½-hour periods of measurement with the different cats are shown in table 1, grouped according to the type of experiment. Under basal conditions the averages for the group varied in the eight periods from 0.76 to 0.77. The widest range in the average values for the individual periods was from 0.74 with A to 0.78 with O and W. There was only a slight tendency toward a fall in quotient in the 4 hours. The average respiratory quotients for 4 hours on the 6 different days of measurement on each cat varied from 0.73 to 0.78 with A, from 0.72 to 0.80 with M, from 0.72 to 0.82 with O, and from 0.73 to 0.81 with W. Variations in the basal values of the R. Q. of cats reported in the literature are somewhat the same as these or greater. The average values in table 1 agree most nearly with those of Ring ('35).

The effect on the R. Q. of the ingestion of water was only slight, on the average. This slight effect was most noticeable in general in the first ½-hour period, although it persisted with G for the entire 4 hours and with W for 3 hours. The other cats showed little or no effect of the ingestion of water. In a few of the individual periods on particular days, the R. Q. values after water were lower than on the basal days.

The effect of the ingestion of 10 gm. of glucose was noticeable in all periods. The average R. Q. for this group of experiments was 0.78 in the first period, and the average peak was 0.89 in the sixth and seventh periods. Both A and G had higher R. Q. values, on the average and in individual experiments, than did the other three cats. These two animals were also the lowest in body weight. In two experiments with cat O and in one experiment with cat A the measurements were continued for 7½ hours. In these particular experiments the maximum R. Q. with A was 0.85 in the fourth, fifth, and sixth periods, but with O the

TABLE 1

Average respiratory quotients of cats as affected by ingestion of sugar.

CONDITION [1] AND CAT (1938-1939)	NO. OF EXPTS.	BODY WEIGHT (KG.)	HALF-HOUR PERIODS AFTER DOSE							
			1 [2]	2	3	4	5	6	7	8
Basal										
A	6	2.8	0.76	0.75	0.76	0.76	0.76	0.76	0.75	0.74
G	3	3.2	0.77	0.76	0.76	0.77	0.76	0.76	0.76	0.75
M	6	4.2	0.75	0.75	0.76	0.76	0.76	0.76	0.77	0.77
O	6	3.9	0.78	0.76	0.77	0.76	0.77	0.77	0.78	0.76
W	6	4.2	0.78	0.77	0.77	0.77	0.76	0.76	0.76	0.75
Water [3]										
A	5	3.1	0.77	0.76	0.77	0.76	0.75	0.75	0.75	0.74
G	2	3.5	0.78	...	0.80	0.79	0.78	0.78	0.78	0.78
M	6	4.3	0.76	0.76	0.77	0.77	0.77	0.77	0.77	0.75
O	6	4.0	0.77	0.75	0.75	0.75	0.75	0.74	0.75	0.73
W	5	4.3	0.82	0.79	0.79	0.78	0.77	0.77	0.76	0.75
Glucose [4]										
A	7	3.3	0.78	0.83	0.87	0.89	0.92	0.91	0.89	0.84
G	3	3.5	0.81	0.87	0.93	0.94	0.96	0.95	0.94	0.93
M	6	4.3	0.77	0.79	0.82	0.83	0.82	0.87	0.87	0.86
O	7	4.0	0.77	0.76	0.80	0.81	0.83	0.85	0.86	0.88
W	6	4.4	0.79	0.78	0.81	0.83	0.85	0.87	0.88	0.88
Fructose [4]										
A	6	3.3	0.76	0.81	0.85	0.86	0.86	0.84	0.82	0.79
G	4	3.5	0.78	0.85	0.87	0.88	0.87	0.87	0.86	0.85
M	6	4.4	0.74	0.77	0.82	0.83	0.83	0.83	0.85	0.82
O	6	4.0	0.77	0.76	0.78	0.79	0.80	0.81	0.82	0.81
W	7	4.5	0.80	0.77	0.79	0.81	0.82	0.81	0.81	0.80
Galactose [4]										
A	5	3.1	0.77	0.81	0.84	0.82	0.84	0.84	0.84	0.83
G	3	3.4	0.84	0.88	0.91	0.91	0.91	0.88	0.87	0.87
M	5	4.2	0.73	0.76	0.80	0.81	0.81	0.82	0.82	0.80
O	5	4.0	0.77	0.77	0.80	0.81	0.81	0.82	0.82	0.81
W	6	4.5	0.81	0.77	0.79	0.81	0.83	0.82	0.83	0.83
Sucrose [4]										
A	2	3.4	0.75	0.78	0.84	0.86	0.86	0.88	0.88	0.87
G	1	3.5	0.86	0.94	0.96	0.96	0.97	0.93	0.95	0.91
Maltose [4]										
A	2	3.5	0.78	0.80	0.85	0.87	0.91	0.90	0.90	0.86
M	3	4.1	0.75	0.75	0.78	0.80	0.80	0.82	0.85	0.84
Lactose [4]										
O	2	3.8	0.74	0.77	0.78	0.79	0.80	0.80	0.78	0.78
W	3	4.4	0.84	0.77	0.81	0.83	0.83	0.84	0.83	0.82
Glucose + galactose [5]										
W	3	4.5	0.80	0.77	0.81	0.84	0.86	0.85	0.88	0.88
Glucose + fructose [5]										
A	2	3.3	0.76	0.82	0.87	0.90	0.94	0.93	0.89	0.84

[1] Without food 16 to 24 hours at start of each experiment.

[2] The first ½-hour period began about 20 mins. after the dose.

[3] 75 ml. of water at 37°C.

[4] 10 gm. of sugar in 50 ml. of water at 37° plus 25 ml. of water for rinse.

[5] 5 gm. of each sugar in the usual amount of water.

maximum values were 0.92 and 0.89 in the tenth period. At the end of fifteen ½-hour periods the R. Q. with A was 0.76 and with O was still 0.84. With cats, therefore, the effect of glucose can last at least 7½ hours.

The effect on the R. Q. of the ingestion of 10 gm. of fructose was noticeable by the second period and, in general, was at its peak (group average, 0.84) in the fifth period. Again the most marked effects were with A and G. In one experiment with O the measurements were continued for 7½ hours. At the end of that time the R. Q. was still 0.79. Hence 10 gm. of fructose may produce a rise in the R. Q. of the cat lasting nearly 8 hours. The rises in value of the R. Q. of these cats after fructose were not so large as those after glucose and were not nearly so large as those noted with man after 50 and 100 gm. of fructose. Also the peak effect with man usually occurs by the end of the first hour.

The rise in R. Q. after the ingestion of 10 gm. of galactose was noticeable in the second period and reached its peak, on the average, in the fifth, sixth, and seventh periods. Again A and G had the greatest rises, and with G the peak was 0.91, which is higher than the peak with this cat after fructose. One experiment with O of 7½ hours' duration gave an R. Q. of 0.80 in the last ½ hour. The rises in the R. Q. values of the cats were, on the whole, not so large nor so prompt as those with man after 50 gm. of galactose.

The ingestion of 10 gm. of sucrose was followed by a rise in R. Q. to 0.97 in the fifth period in one experiment with G and to an average of 0.88 in the sixth and seventh periods in two experiments with A. On the whole, when the experiments are assessed individually, it is evident that the rise was not so large as with glucose with either animal.

Maltose produced a greater rise in R. Q. in two experiments with A than did sucrose, although the rise was not so large as with glucose. In three experiments with M there was a rise in quotient with maltose but not so large as that with glucose.

The ingestion of 10 gm. of lactose was followed by a rise in R. Q. to an average maximum of 0.80 in the fifth and sixth periods in two experiments with O, and to 0.84 in the sixth period in three experiments with W. In the individual experiments with W there were maximum values of 0.88 and 0.87 in the sixth period. The general average for the three experiments with W is lowered by one experiment in which the average R. Q. for 4 hours was 0.78.

In three experiments with W with 5 gm. of glucose plus 5 gm. of galactose (approximately 10 gm. of lactose), the rise was as large as with glucose alone and larger than with galactose alone. Therefore, as has been found by others (Folin and Berglund, '22; Bodansky, '23; Harding and Grant, '33), the addition of glucose to galactose improved the metabolism of galactose.

The ingestion of an approximate equivalent to 10 gm. of sucrose (5 gm. each of glucose and fructose) in two experiments with A was followed by an average rise in R. Q. to 0.94 in the fifth period, which is even greater than the rise after ingestion of 10 gm. of glucose by the same animal. The effect of the combination of sugars was greater than that of 10 gm. of sucrose alone.

Total metabolism and carbohydrate metabolized

The average values for the total volumes of carbon dioxide eliminated and oxygen absorbed in the various groups of experiments with each cat are given in table 2. In general, these average values were lower under basal conditions than under the other conditions. No attempt has been made to assess the effects of ingestion of the sugars on the metabolism of energy. In most of the experiments the cats were quiet for the greater part of the time, but occasionally there were periods of marked activity. Study of the original protocols shows that it would be difficult to assess the values in relation to relative activity. Hence it seems best to report the total measurements as made, rather than to select periods arbitrarily and empirically and try to interpolate for the periods rejected. The values are therefore given as measured and can be used for purposes of calculation by other workers.

The main purpose of this study was to determine the effects of ingestion of various sugars upon the R. Q. and the metabolism of carbohydrates. Calculation of the amount of carbohydrate metabolized might be carried out simply by apportioning the metabolism between that of carbohydrate and that of fat, according to the total respiratory exchange. This method of calculation, however, would give too high values, because the metabolism of protein was not considered. Usually it is estimated that from 10 to 15% of the total energy output is derived from the metabolism of protein. As the cat is a carnivorous animal, it would under natural conditions have a predominantly protein metabolism. These animals were fed on canned salmon, mackerel, liver, beef, and milk. Therefore the proportion of the total metabolism represented by the protein metabolism must have been higher than

TABLE 2

Total metabolism, R. Q., and calculated carbohydrate metabolism of cats as affected by ingestion of 10 gm. of sugar.

(Average values per 4 hours)

CAT AND CONDITION	TOTAL METABOLISM		R. Q.		CARBOHYDRATE METABOLIZED
	CO_2	O_2	Total	Non-protein [1]	
	liters	*liters*			*gm.*
A					
Basal	3.92	5.20	0.75	0.71	0.0
Water	4.40	5.81	0.76	0.71	0.0
Glucose	4.75	5.49	0.87	0.92	2.4
Fructose	4.70	5.72	0.82	0.84	1.5
Galactose	4.72	5.72	0.83	0.84	1.6
Sucrose	4.14	4.93	0.84	0.88	1.5
Maltose	4.38	5.11	0.86	0.91	2.0
Glucose + fructose [2]	4.45	5.11	0.87	0.94	2.2
G					
Basal	4.95	6.51	0.76	0.71	0.0
Water	5.44	6.94	0.78	0.71	0.0
Glucose	7.02	7.68	0.91	1.13	4.9
Fructose	6.49	7.61	0.85	0.95	2.3
Galactose	6.62	7.49	0.88	1.05	3.7
Sucrose	7.09	7.60	0.93	1.20	5.2
M					
Basal	5.84	7.69	0.76	0.71	0.0
Water	5.69	7.46	0.76	0.71	0.0
Glucose	5.64	6.79	0.83	0.86	1.8
Fructose	5.99	7.39	0.81	0.81	1.4
Galactose	5.91	7.45	0.79	0.78	0.9
Maltose	4.85	6.07	0.80	0.78	0.6
O					
Basal	5.39	7.02	0.77	0.71	0.0
Water	5.29	7.05	0.75	0.71	0.0
Glucose	5.45	6.65	0.82	0.83	1.8
Fructose	5.80	7.31	0.79	0.78	1.3
Galactose	5.62	7.03	0.80	0.79	1.4
Lactose	4.75	6.09	0.78	0.75	0.6
W					
Basal	4.57	6.00	0.76	0.71	0.0
Water	5.34	6.85	0.78	0.71	0.0
Glucose	5.81	6.96	0.83	0.89	1.6
Fructose	6.10	7.61	0.80	0.79	0.9
Galactose	6.01	7.47	0.81	0.80	1.0
Lactose	5.59	6.81	0.82	0.85	1.2
Glucose + galactose [2]	5.51	6.59	0.84	0.91	1.5

[1] The average calculated protein combustion per 4 hours in basal and water expts., respectively, was: A, 2.37 and 2.84 gm.; G, 3.39 and 5.39 gm.; M, 4.05 and 4.11 gm.; O, 4.24 and 2.97 gm.; W, 3.27 and 4.97 gm.

[2] 5 gm. of each sugar.

15%. For purposes of calculation of the comparative amounts of carbohydrate metabolized after ingestion of the different sugars, the following assumptions have been made.

In the basal experiments and in the control experiments with water, as the R. Q. values were below 0.81, it has been assumed that the energy was derived from the metabolism of fat and protein alone. The average values for the protein combustion per 4 hours in the basal and the water experiments, as calculated on the basis of this assumption, are given in footnote 1 in table 2. It is recognized that they are wholly hypothetical and may not correspond to the facts. However, in view of the lack of information regarding the urinary nitrogen, the average value for each cat for the protein metabolized in the water experiments with this cat has been used as the basis of calculating the values of the non-protein R. Q. in the other groups of experiments with the same cat. The amounts of carbohydrate metabolized (table 2) have been calculated in the usual empirical manner from the non-protein gaseous exchange. In the three instances where the non-protein R. Q. values were greater than 1.00 (G after ingestion of glucose, galactose, and sucrose), the carbohydrate converted into fat has also been included in the values given in table 2, the calculation being made according to the method proposed by Lusk ('28).

Among the monosaccharides, glucose caused the greatest metabolism of carbohydrates in all five cats, galactose was next in order with four out of five cats, and fructose in general caused the least metabolism of carbohydrates. The disaccharides gave values that were sometimes higher and sometimes lower than the sum of the values for 5 gm. each of their constituent hexoses. The combination of glucose with one of the other hexoses to form a total amount of sugar equivalent to 10 gm. of sucrose or lactose gave results that were higher than would be expected by calculation of the sums of the effects of the sugars separately. This latter phenomenon was also observed with man after hexoses ingested separately and together.

In most of the experiments less than half of the 10 gm. of sugar was metabolized by any cat in the 4 hours of observation. Indeed, at the rate at which they were metabolized, many of the sugars would have supplied the metabolism of carbohydrates for over 24 hours. The serial order of the amounts of carbohydrate metabolized after the ingestion of these different sugars would be the same, regardless of the level of the metabolism of protein. Therefore the values given in table 2 serve for comparative purposes. It is apparent that cats have the ability to metabolize the disaccharides nearly as well as the mono-

saccharides, although two of the disaccharides, sucrose and maltose, are not common articles in the cat's diet in the natural state.

SUMMARY

The respiratory exchange was measured in successive ½-hour periods for 4 hours with five cats in the basal state, after ingestion of 75 ml. of water, after ingestion of 10 gm. of glucose, fructose, galactose, sucrose, maltose, or lactose, and after ingestion of a combination of 5 gm. each of glucose and fructose or of glucose and galactose.

The values of the basal R. Q. were uniform, for the most part, and did not show a marked tendency to change during the eight ½-hour periods of measurement. The ingestion of water resulted in a rise in R. Q. with one cat for the entire 4 hours but with the other cats only in the first ½-hour period. Glucose caused the greatest rise in R. Q., and the peak occurred in the sixth and seventh ½-hour periods. All the other sugars, disaccharides as well as monosaccharides, caused definite rises in the R. Q.

On the assumption that in the control experiments with water only fat and protein were metabolized and that in the experiments with the sugars the protein metabolism of a given cat was the same as its average protein metabolism in the experiments with water, it was calculated that the metabolism of carbohydrates was highest after glucose and lower after galactose and fructose in the order named. The cats were able to metabolize the disaccharides nearly as well as would be expected, in view of their constituent monosaccharides formed by hydrolysis. When combinations of hexoses equivalent to 10 gm. of sucrose or lactose were ingested, the resultant metabolism of carbohydrates was greater than would be expected from the sum of the amounts metabolized after ingestion of the respective hexoses given separately.

Cats resemble men in the metabolism of the monosaccharides in that they show increases in R. Q. and in carbohydrate metabolism after ingestion of these sugars, but they differ from men in that the peak effect does not occur so promptly and, qualitatively, the order of magnitude of the effect is not the same.

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THE AMINO ACID REQUIREMENTS OF THE CHICK

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THREE FIGURES

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Previous studies of the amino acid requirements of the chick have been carried out by using diets containing ingredients which are poor sources of a certain amino acid, or by removing one or more of the amino acids after acid hydrolysis. By using these methods, it has been shown that the chick must have dietary sources of arginine, glycine, histidine, tryptophane, lysine and methionine (see review by Almquist, '42, also Grau and Almquist, '43) and isoleucine (Grau and Almquist, '44). In a continuation of these studies, mixtures of pure amino acids have been used in complete replacement of all proteins in the diet. Some of the amino acids which have already been shown to be required by the chick (glycine, histidine and isoleucine) have been reinvestigated with diets containing the amino acid mixtures; the other recognized amino acids which have been studied include alanine, aspartic acid, glutamic acid, hydroxyproline, leucine, norleucine, phenylalanine, proline, serine, threonine, tyrosine and valine. The tryptophane requirement will be discussed elsewhere.

EXPERIMENTAL

White Leghorn chicks, which had been reared on a commercial-type chick mash for 10–14 days, were maintained as reported previously (Grau and Almquist, '43). The basal ration was as follows: cellulose [1] 5, calcium gluconate 8, mineral mixture 3.24, crude soybean oil 5, sardine oil (400D–3000A per gram) to which 1% of mixed tocopherols [2] had been added 0.25, choline chloride 0.2, inositol 0.1, cholic acid 0.1, 2-methyl-1, 4-naphthohydroquinone diacetate 0.001, thiamine 0.001, riboflavin 0.001, pyridoxine 0.001, nicotinic acid 0.003, calcium pantothenate *(dl)* 0.003, biotin (acid)[3] 0.00001 gm., with the amino acid mixture and glucose [4]

[1] Cellu Flour.

[2] Natural Mixed Tocopherols, Type I (Distillation Products, Inc.).

[3] The biotin was kindly provided by Merck and Co., Inc. through the courtesy of Dr. J. C. Keresteszy.

[4] Cerelose.

to make 100 gm. Other members of the vitamin B-complex were provided by a concentrate prepared from solubilized liver which added only 4 mg. nitrogen to each 100 gm. diet. The mineral mixture furnished the following: tricalcium phosphate 2000, dipotassium phosphate 500, potassium chloride 300, and manganese 10, silicon 46, magnesium 48, aluminum 8, iron 14, copper 1, zinc 1, iodine 0.8 and cobalt 0.5 mg. per 100 gm. diet.

TABLE 1

Mixture of amino acids used in the positive control diet.

AMINO ACID	FORM OF ACID	LEVEL AS FED	LEVEL OF NATURAL ISOMER	AMINO ACID	FORM OF ACID	LEVEL AS FED	LEVEL OF NATURAL ISOMER
		%	%			%	%
Alanine	dl	1.0	0.5	Methionine	dl	1.0	0.5
Arginine	l(+)·HCl	1.4	1.2	Norleucine	dl	0.2	0.1
Aspartic acid	l(+)	2.0	2.0	Phenylalanine	dl	1.0	0.5
Cystine	l(−)	0.4	0.4	Proline	l(−)	2.0	2.0
Glutamic acid	l(+)	5.0	5.0	Serine	dl	0.4	0.2
Glycine	....	1.8	1.8	Threonine	dl	3.0	1.5
Histidine	l(+)·HCl·H_2O	0.8	0.6	Tryptophane	l(−)	0.4	0.4
Hydroxyproline	l(−)	0.2	0.2	Tyrosine	l(−)	2.0	2.0
Isoleucine	dl	2.0	1.0	Valine	dl	2.0	1.0
Leucine	l(−)	2.0	2.0		Total	30.0	24.0
Lysine	l(+)·HCl	1.4	1.1				
				(Sodium bicarbonate)		1.5	

The amino acids used in these studies were commercial products. The levels used in the diets and the form in which they were added are summarized in table 1. Glucose replaced the omitted amino acids except for the glutamic acid studies, where aspartic acid was added. The sodium bicarbonate was added to neutralize the hydrochlorides of the basic amino acids and to provide sodium chloride, which was omitted from the mineral mixture generally used.

RESULTS

Alanine. Four chicks maintained for 10 days on a diet lacking alanine gained in weight just as well as a similar group which was fed alanine. Alanine is apparently completely dispensable for chick growth.

Aspartic acid. In the first experiment dealing with this amino acid, glutamic acid as well as aspartic acid was omitted, and no attempt was made to replace this loss of amino acid nitrogen. This group of four chicks lost weight for 2 days, then began to gain at an increasing rate, so that from the time of minimum weight until 10 days later, the gain

per day [5] was 2.4%. Over a similar period, the positive control group gained at a rate of 4.0%. In a later experiment, where aspartic acid alone was omitted, the growth rate obtained with three chicks for 14 days was the same as that of the positive control. These data indicate that aspartic acid is a dispensable component of the diet of the chick.

Glutamic acid. Besides the experiment mentioned above, in which both of the dicarboxylic acids were omitted, three chicks were kept for 14 days on a diet containing extra aspartic acid, but no glutamic acid.

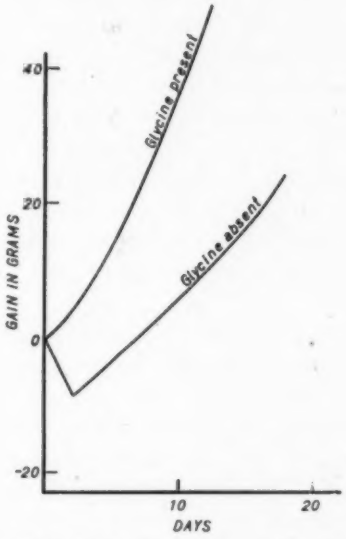

Fig. 1 Effect of omission of glycine from the amino acid mixture on the growth of chicks.

The total *l* (+)- aspartic acid content of the diet was 4.0% instead of the usual 2.0%. These chicks reacted in the same way as those which received neither aspartic acid nor glutamic acid, showing an original weight loss, and a slow gain thereafter. Because of the indecisive nature of these results, it can only be said that lack of glutamic acid causes slower gains than those obtained with control groups, and that if glutamic acid is required at all in the diet, it is needed only to promote most efficient growth.

Glycine (fig. 1). That the chick is able to carry on definite, but limited glycine synthesis was suggested when casein diets containing low levels of glycine gave a moderate growth which was increased markedly by

[5] Average per cent gain per day = $\frac{\text{Average gain} \times 100}{\text{No. days on expt.} \times \text{avg. wt. during expt.}}$

additions of glycine to the diet (Almquist and Mecchi, '42). Results with four chicks on a glycine-deficient amino acid mixture for 18 days showed poor growth and gain per gram of food eaten. Omission or inclusion of serine had no effect on the glycine deficiency. These results with diets free from glycine confirm the previous conclusion that the chick can synthesize glycine, but not at a rate high enough to allow best growth.

Histidine. Four chicks which received a histidine-deficient diet lost weight for the first 4 days of the experiment and remained at this low weight level for the remainder of the 10-day period. Other chicks which received 0.15% *l* (+)- histidine (fed as the monohydrochloride monohydrate) showed gains which indicated that most of the requirement for this amino acid had been met. Histidine is, therefore, an indispensable amino acid.

Isoleucine. A group of three chicks fed a diet deficient in isoleucine lost weight and died, confirming the previous investigations in which dried beef blood cell protein was used as the sole protein source (Grau and Almquist, '44), and in which the necessity of isoleucine in the diet was proved.

Leucine. A preliminary experiment with three chicks indicated that chicks lost weight rapidly on leucine-deficient diets, and that while either 4.0% of the *dl* form or 2.0% of the *l* (−) form was adequate, 1.0% of the *l* (−) form was inadequate for best growth. Another experiment with three chicks maintained for 16 days showed a rapid weight loss followed by a more gradual loss; the curve was practically identical with that obtained with phenylalanine or threonine deficiencies (figs. 2 and 3). Leucine is an indispensable component of the diet.

Norleucine. Omission of norleucine from the diet appeared to have no adverse effect on the rate of gain of three chicks which were maintained for 16 days. This amino acid is apparently completely dispensable for the chick.

Phenylalanine. Four chicks on a phenylalanine-deficient diet lost weight rapidly (fig. 2) when the diet contained 2.0% *l* (—)- tyrosine, but gained at the normal rate when 1.0% *dl*-phenylalanine was present in addition. Indicated interrelationships between these two amino acids are discussed under tyrosine.

Proline and hydroxyproline. These amino acids were omitted from the diet of four chicks to determine if either one was necessary. The chicks maintained their weight for the first 4 days, but did not grow; after that time, they grew at the normal rate. This apparent time lag may have been required to allow establishment of adequate synthesis of

these amino acids from others furnished in the diet. In any case, the omission of the prolines had no lasting effect on the growth rate; hence, they may be classified as dispensable amino acids.

Serine. Unlike glycine, serine is a completely dispensable component of the diet of the chick, as was shown in the normal growth of four chicks maintained for 8 days on a serine-deficient ration. As mentioned above, serine has no ability to replace glycine as an essential dietary component.

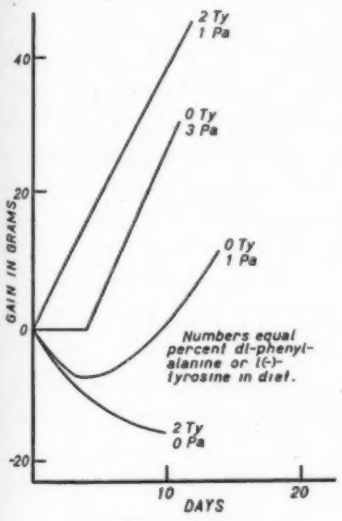

Figure 2

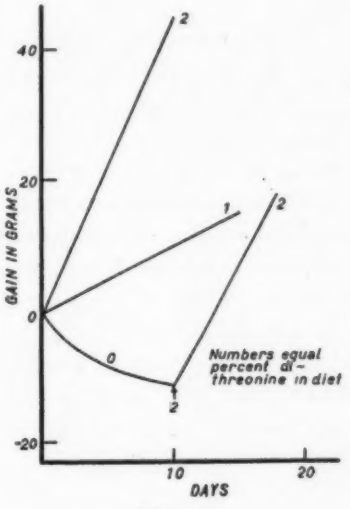

Figure 3

Fig. 2 Curves showing the effects of phenylalanine and tyrosine deficiencies on the growth of chicks.

Fig. 3 Effect of threonine deficiency on chick growth.

Threonine. Some of the results with threonine are shown in figure 3, where the recovery from an amino acid deficiency is shown to occur immediately after supplying the missing substance. The change from weight loss to gain in 24–48 hours is often a clear indication of the future course of such an experiment. This early weight gain is primarily a reflection of the increased appetite which is caused by the change in diet: fresh diet alone will not evince the effect. Feeding the *dl* form at 1.0% of the diet evidently is not enough for best growth, and, while 2.0% appears adequate, 3.0% was fed for safety until the most satisfactory level could be determined. In another experiment with two chicks, a threonine-deficient diet was fed for 27 days, at which time

one of the chicks died, with no apparent symptoms other than those which would occur with malnutrition or starvation.

Tyrosine. The absolute status of tyrosine cannot be determined from the data to be presented here, but must await further study. As shown in figure 2, addition of tyrosine is required to allow best growth if only 1.0% of the *dl*-phenylalanine is present in the diet, but 3.0% *dl*-phenylalanine alone appears to obviate the need for additional tyrosine. Tentatively, then, it may be said that tyrosine is required to promote optimal growth if phenylalanine is present at only a moderate level in the diet.

Valine. Of all the deficiencies studied, valine and isoleucine seem to exert the most profound effects on chicks; however, none of the peculiarities of gait or sensitiveness to touch, such as were observed with rats (Rose, '38), have been noted. The rapid and continuing decline in weight with valine deficiency was almost identical with that observed with phenylalanine deficiency (fig. 2). Three of the four chicks on one valine-deficient diet died on the twelfth day of the experiment, and all were extremely weak for a few days before this time. Addition of either 2.0% isovaline (*dl*-α-amino-α-methylbutyric acid) or 2.0% norvaline (*dl*-α-amino-n-valeric acid) did not diminish the severity of the deficiency. After 6 days, *dl*-valine was added. Within 24 hours, all chicks had gained in weight, and they continued to do so until the experiment was terminated 8 days later.

SUMMARY

Mixtures of 20 amino acids have been used in complete replacement of protein in chick diets. Of these amino acids, alanine, aspartic acid, hydroxyproline, proline, norleucine and serine have been shown to be dispensable. The essential nature of glycine in promoting best growth has been confirmed; glutamic acid appears similar to glycine in this regard. Tyrosine appears to be required if only moderate levels of phenylalanine are used, but seems to be dispensable when higher levels are used. Leucine, phenylalanine, threonine and valine have been shown to be necessary dietary components to prevent weight loss, while the similar status of histidine and isoleucine has been confirmed.

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DENTAL CARIES IN THE COTTON RAT[1]

I. METHODS OF STUDY AND PRELIMINARY NUTRITIONAL EXPERIMENTS

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ONE FIGURE

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The vitamin B complex requirements of the cotton rat (Sigmondon hispidus hispidus) have been described by McIntire, Schweigert and Elvehjem ('44). In the course of the experiments in which these requirements were evaluated, a preliminary survey was made to determine how susceptible the teeth of the cotton rat were to decay. It was found that the molar teeth of the cotton rats which were raised from weaning on synthetic, high sucrose rations were highly susceptible to decay. On the contrary, the molar teeth of cotton rats which were on stock rations composed of crude materials were highly resistant to decay. Therefore, the various experiments reported here were devised to study the factors involved in the incidence and progress of the carious lesions in the molars of the cotton rat.

EXPERIMENTAL

The cotton rats used in these experiments were obtained from our stock colony which was maintained on the Steenbock stock ration no. 14[2] supplemented with greens. These animals were weaned at the age of 3 weeks and at a weight of 20 to 25 gm.

The basal ration (801) consisted of: Labco casein 18%, sucrose 73%, salts IV 4% (Phillips and Hart, '35) and corn oil 5%. To each 100 gm. of ration was added: thiamine 250 μg., riboflavin 300 μg., nicotinic acid

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[2] Ground corn 71.5%, linseed oil meal 15.0%, alfalfa leaf meal 2.0%, $CaHPO_4$ 1.0%, NaCl 0.5%, crude casein 5.0% and butter fat 5.0%.

2.5 mg., pyridoxine 250 μg., pantothenic acid 2 mg., choline 100 mg., inositol 100 mg., and p-aminobenzoic acid 30 mg.; 1 drop of haliver oil was fed weekly. Later a modification (802) of the basal ration was found to give more uniform growth. It consisted of an increase in the casein to 24% at the expense of the sucrose.

In early experiments the cotton rats were left on experiment for periods ranging from 10 to 14 weeks to determine the optimum experimental period. For accurate results it was found necessary to sacrifice the animals at a time when the carious lesions were large enough to be readily detected and yet not so large that any extensive amount of fracturing of the cusps had occurred. It is especially necessary to have a minimum amount of fracturing. Old widespread fractured areas may overlie and involve several of the original carious lesions. Thus any accurate evaluation of the numbers of lesions and the extent of each lesion would be impossible. In preliminary studies it was found that the most suitable experimental period lay between 10 to 14 weeks after weaning. When 14 weeks was established as the optimum period, all animals were sacrificed at that age.

Various liver preparations (1:20 liver extract, alcohol ether extract of 1:20 liver extract, solubilized liver, and whole liver substance) were added to the basal ration to determine whether the more rapid rate of growth thus obtained would have any effect upon the incidence and progress of carious lesions.

In order to determine whether or not additional fat-soluble vitamins would have any effect on the development of the carious lesions, these vitamins were fed to one group at the following levels per 100 gm. of ration: 500 μg. of β-carotene, 12.5 μg. of calciferol, 2 mg. of α-tocopherol, and 187.5 μg. of 2 methyl, 1, 4 naphthoquinone. These vitamins were kept in chloroform in the refrigerator. When the ration was to be mixed, the necessary amount of the chloroform solution was evaporated to dryness and the vitamins taken up in corn oil so that 5 gm. of corn oil contained the amount of these vitamins to be added to 100 gm. of ration. This corn oil was then incorporated into the ration in the customary fashion.

In order to compare the effect of particle size of the diet upon the incidence of carious lesions two procedures were followed. The first comparison was made with Steenbock stock ration no. 14 in which the particles of corn used in its composition would pass through a 20 mesh, but not through a 40 mesh sieve. A portion of this ration was then ground in a ball mill until all would pass through a 60 mesh sieve. The coarse ration was fed to one group, while the fine was fed to a second

group. The second comparison was made by the substitution of dextrin for sucrose in ration 801. The particle size of dextrin used in the coarse ration was such that it would pass through a 20, but not through a 40 mesh sieve; and that in the fine ration would all pass through a 60 mesh sieve.

At the end of the experimental period, the animals were sacrificed. The heads were fixed in 95% ethanol for 24 to 48 hours. They were then skinned and the flesh removed. The jaws were placed in fresh 95% ethanol and stored until observations could be made. The observations on the molar teeth reported here were made upon successive planes ground parallel to the occlusal surface by means of a vertically rotating carborundum wheel. After each grinding, the newly exposed plane was observed at a magnification of 30 diameters with a binocular dissecting microscope. Each lesion observed was carefully explored with a no. 42 fine, smooth dental broach to determine the depth of softening, as a supplement to the visual changes in color and opalescence.

RESULTS

Description of the dentition of the cotton rat

The dentition of the cotton rat is similar to that of the white rat: monophyodont, one incisor and three molars in each quadrant of the jaws, continuously erupting incisors, and molars which are more highly developed than the incisors and which are limited in development to the early period of life. The molars are similar to those of the white rat in most respects, but have a few notable differences. When the molars of the cotton rat are erupted, there are definite cusps as in the white rat. But in the cotton rat the completely transverse occlusal fissures between the cusps are very shallow. Attrition of the cusps exposes small circular areas of dentin which become larger as the cusp is more deeply abraded. Since enamel is more resistant to attrition, the enamel rim is perceptibly higher than the exposed dentin.

After a period of 3 or 4 weeks of occlusal contact, sufficient attrition has occurred that the cusps have been abraded to a level below the bottom of the shallow transverse occlusal fissures. Then all the cusps of any molar become connected at the occlusal surface. The exposed dentin area of a cusp is connected to the similar area in the next anterior or posterior cusp by a narrow strip of exposed dentin, flanked on each side by enamel. Thus in the cotton rat, a few weeks after the eruption of any molar, its occlusal surface has a continuous region of exposed dentin extending from the anterior cusp to the posterior cusp. This can be

seen in figure 1 (except in the third molars of the upper jaw which are not fully erupted).

The occlusal fissures which are present in the fully erupted molar extend from ⅓ to ⅔ across the tooth from either the labial or lingual surface. These fissures are exceedingly narrow and extend from the occlusal surface of the molar almost to the cemento-enamel junction.

Regions of tooth decay

In the cotton rat it was found that tooth decay occurred almost exclusively in the occlusal fissures and at such a depth that early decay could not be observed in the intact tooth because of the depth and narrowness of these fissures. When decay had progressed sufficiently, regions of the cusps were broken away due to the undermining effect of these deeply set carious regions. Only then, was it possible to determine from the occlusal surface that decay had been occurring in the occlusal fissures. Hence, in order to study and evaluate decay in the teeth of the cotton rat, it was necessary to use an alternate grinding and observation procedure similar to that suggested by Cox and Dixon ('39).

A very low incidence of decay was found on the proximal surfaces of the molars or on the exposed occlusal surfaces. The rarest location where decay was found was along the gingival margin. However, where decay was found in these regions the development and the progress of the lesions was entirely comparable to the lesions found in the narrow occlusal fissures.

The first indication of decay was a slight darkening of the enamel, and a decrease in its opalescence. This was accompanied by a softening of the affected area. This early lesion became quite evident when it was still less than 0.1 mm. in diameter. At that time the enamel was softened sufficiently that a no. 42 fine, smooth dental broach would readily penetrate a short distance into the lesion. The destruction of the enamel apparently was quite rapid once the lesion had been initiated. Frequently an area 0.2 to 0.3 mm. in diameter would have become decayed before any appreciable amount of penetration into the dentin had occurred. Once the dentin had become affected the lesion spread more rapidly until large areas of the cusp were involved. About this time fracture of the undermined crown of the cusp began. Fracture of large portions of the cusp did not usually occur immediately but instead small portions broke off as they were undermined. There is ample evidence for this statement since many lesions occurred where there was a loss of only small portions of a cusp due to fracture. Fracture continued progressively as decay progressed until finally the entire

portion of the cusp affected was undermined and broken off; after fracture occurred, decay continued in the original lesion at about the same rate. The surface exposed by the fracture did not appear to be attacked by the process of decay at any greater rate than if fracture had not occurred. Instead decay progressed into this exposed face from the original lesion as if no fracture had occurred.

The method of recording position and evaluating the extent of the carious lesions

In order to record the number of decayed areas in such a way as to be able to study the frequency in any given area, the various occlusal fissures were numbered in a manner similar to that developed by Cox, Dodds, Dixon and Matuschak ('39) for use in the evaluation of corn meal caries in the white rat. It was not possible to use the identical numbering system of Cox et al. since the cotton rat differs from the white rat in too many anatomical details. Figure 1 shows the numbering system used for the cotton rat.

An arbitrary system for the evaluation of the extent to which the decay had progressed in any lesion was devised. The system employed symbols 1 + to 5 + to represent the increasing extent of the lesions, as follows: 1 + — a lesion where there was a small, dark, definitely softened decayed region in the enamel without any penetration of the dentin; 2 + — a lesion with a more widespread softening of the enamel and a slight penetration into the dentin; 3 + — a lesion with widespread decay of the enamel and deep penetration of the dentin but without fracture; 4 + — a lesion with widespread decay in both the enamel and the dentin and a small amount of fracture of the undermined cusp; 5 + — a lesion which has progressed so far that a large amount, or all, of the undermined portion was broken off.

For each cotton rat studied the evaluations assigned for the individual carious lesions were totaled. This sum was used as an index of the extent to which decay had progressed in each animal. The average amount of decay for each carious lesion was obtained by dividing this sum by the number of lesions observed.

This type of evaluation of the extent of the lesions was at best an arbitrary one, but a very essential one. There could be no absolute unit of measurement for the progress of decay. Any evaluation assigned by the observer was, of necessity, a measurement of degree which took into account the numerous changes observed in color, texture, and size of the lesions. Therefore, by the system outlined, we have a satisfac-

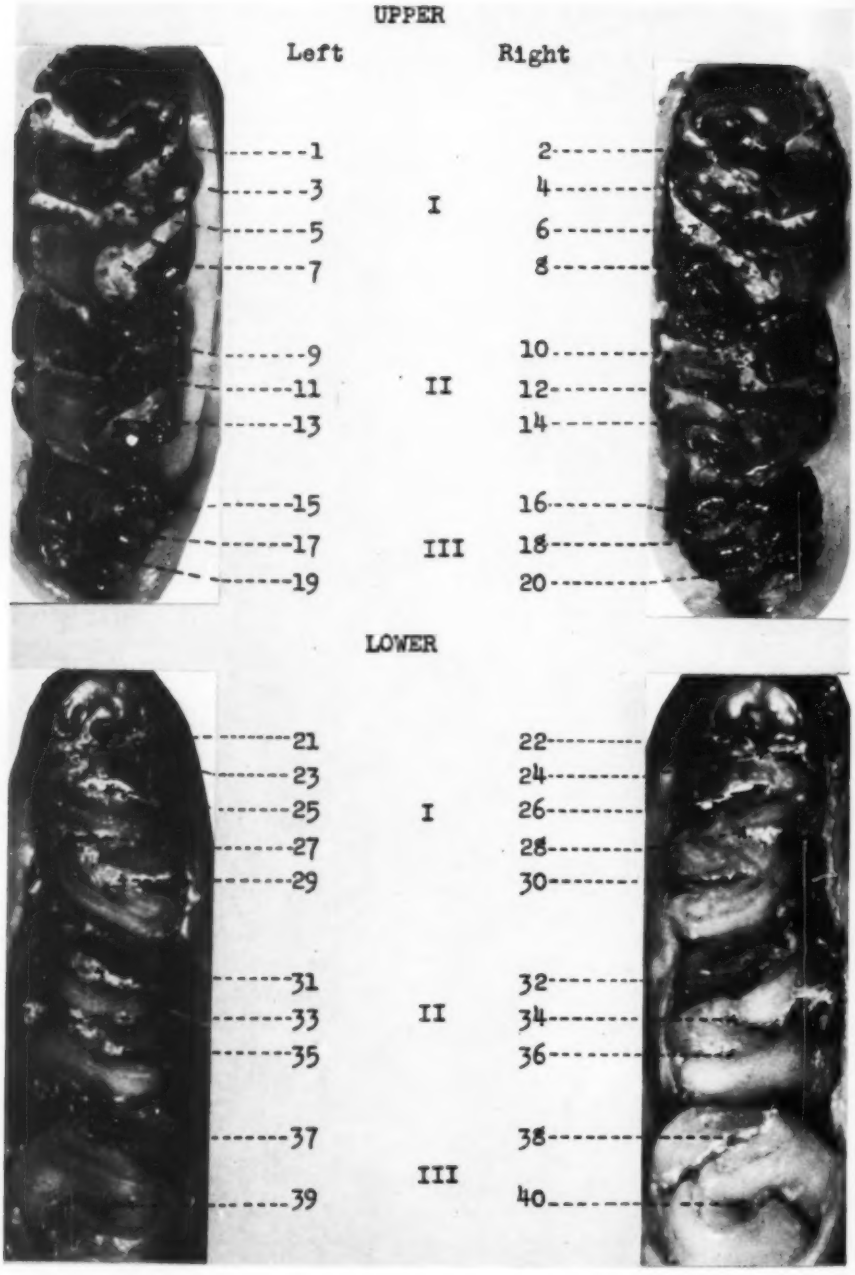

Fig. 1 Occlusal surfaces of the molars of a cotton rat labelled to indicate the occlusal fissures in which the carious lesions occur. This rat was one which had been raised on the stock ration and was entirely free of tooth decay. × 16.

tory and consistent method of evaluating the degree of the carious processes in any animal or group of animals.

The effect of the length of experimental period upon the development of the carious lesions

The observations from the cotton rats which were sacrificed at 10, 12, and 14 weeks to determine the most suitable length of experimental period are presented in table 1. As the duration of the experimental period was increased, there was observed in the cotton rats on each ration, a definite increase in the number of carious lesions per animal, a definite increase in the total extent of the carious lesions and a definite

TABLE 1

The effect of the length of experimental period upon the development of carious lesions in the cotton rat.

RATION	NO. OF WEEKS	NO. OF ANIMALS	AVERAGE NUMBER OF CARIOUS LESIONS	AVERAGE EXTENT OF CARIOUS LESIONS	AVERAGE NUMBER OF CARIOUS LESIONS		AVERAGE EXTENT OF CARIOUS LESIONS	
					Upper jaw	Lower jaw	Upper jaw	Lower jaw
801	10	5	11.8	25.8 +	2.8	9.0	5.8 +	20.0 +
	12	3	21.3	53.0 +	8.6	12.7	16.7 +	36.3 +
	14	2	24.5	78.0 +	10.0	14.5	27.5 +	50.5 +
801 + 4% 1:20 L. E.	10	4	12.0	25.3 +	3.0	9.0	5.5 +	19.8 +
	14	7	26.0	74.3 +	12.1	13.9	32.0 +	42.3 +
802	12	5	22.8	42.2 +	9.8	13.0	19.0 +	23.2 +
	14	4	27.3	63.5 +	12.5	16.0	22.5 +	41.0 +
Coarse stock	12	3	6.0	12.0 +	0.7	5.3	0.7 +	11.3 +
	14	4	6.8	15.5 +	2.0	4.8	2.0 +	13.8 +

increase in the average extent of decay in each carious lesion. It appeared that under our present experimental regime the optimum experimental period was 14 weeks. At that age the carious lesions in the molars had progressed to the point where they were readily visible when exposed by grinding. Yet only a small amount of fracture had occurred. If the experimental period were longer the fracturing would have become so extensive that many individual lesions would have been obscured.

The distribution of the carious lesions in the various molars

In these early experiments there was noted a very conspicuous difference between the incidence of carious lesions in the upper and lower

jaws. In figure 1 it is seen that in both the upper and lower jaws there are 10 occlusal fissures in the three molars of each quadrant. Since, as we have reported above, it was in the occlusal fissures that almost all decay was initiated in the cotton rat molars, each quadrant had an equal number of susceptible regions. However, it was found throughout all the groups of animals studied that there was a much lower incidence of carious lesions and a smaller amount of decay in each lesion in the molars of the upper jaw than in those of the lower jaw (table 1). Once initiated the rate of development of the carious lesions in the molars of the upper jaw appeared to be comparable to that in the lower jaw.

TABLE 2

A summary of the relative incidence and extent of carious lesions in the molars of 65 cotton rats which had been maintained on sucrose rations for 14 weeks.

JAW	MOLAR	TOTAL NUMBER OF CARIOUS LESIONS (A)	TOTAL EXTENT OF CARIOUS LESIONS (B)	AVERAGE EXTENT OF EACH CARIOUS LESION $\left(\frac{B}{A}\right)$	NUMBER OF CARIOUS LESIONS PER MOLAR $\left(\frac{A}{2 \times 65}\right)$	NUMBER OF SUSCEPTIBLE REGIONS PER MOLAR (C)	PER CENT OF SUSCEPTIBLE REGIONS THAT DEVELOPED CARIOUS LESIONS $\left(\frac{A}{2 \times 65} \times \frac{1}{C} \times 100\right)$
Upper	First	122	194 +	1.6 +	.9	4	23
	Second	299	797 +	2.7 +	2.3	3	77
	Third	200	436 +	2.2 +	1.5	3	51
Lower	First	305	632 +	2.1 +	2.3	5	47
	Second	326	952 +	2.9 +	2.5	3	84
	Third	239	737 +	3.1 +	1.8	2	92

Since the lesions in the upper jaw were initiated slightly later than those in the lower jaw, the extent of decay in each of these lesions was always slightly less than in those in the lower jaw at any given time of observation.

Very definite differences were seen in the rate of incidence of carious lesions in the three molars of both the upper and lower quadrants. A summary was made of the number of carious lesions observed in each molar of 65 cotton rats which had been maintained on synthetic rations for 14 weeks. The results of this summary are presented in table 2. It was found that there was an increasing rate of incidence and also an increasing extent of decay in each lesion of the molars in the following order: upper first, lower first, upper third, upper second, lower second, and lower third. The upper molar in each case had a lower rate of incidence of carious lesions and each lesion had a lesser extent of decay than the respective lower molar.

Since the first molars were fully erupted and the second molars almost fully erupted at the beginning of the experiment, they were subjected to the conditions of the oral cavity for about the same length of time. Therefore, if all the factors influencing decay were the same, the first and second molars of all four quadrants would be equally affected. However, from the summary it can easily be seen that there was a very much higher rate of incidence of carious lesions in the second molars than in the first molars of the upper and lower jaws. This different rate of incidence may be affected by one or more of several factors: the structure and topography of the molars themselves and the position of the molars in the mouth.

The lower third molar was erupted after the animals had been on experiment for about 7 weeks. The upper third molar was erupted after about 10 weeks of the experimental period had elapsed. The time of eruption of the upper and lower third molars varied with the rate of growth. The more nearly complete the ration was nutritionally, the more rapidly the animals grew and the sooner the upper and lower third molars were erupted. However, the lower third molars were only exposed to the environment of the oral cavity for about one-half and the upper third molars for only about one-third of the experimental period. The very high incidence of carious lesions in the lower third molars had added significance when this short period of exposure was considered. Although the rate of incidence of carious lesions in the upper third molars was lower than in the lower third molars, the very short period of exposure indicated a high susceptibility to decay. It appeared that the third molars were much more susceptible to decay in the cotton rat than either the first or the second molars. This may be due to the relative position in the mouth, or the structure of the third molars may have been unfavorably affected during the development by some deficiency or deficiencies in the experimental rations.

The effect of diet upon the rate of incidence and the extent of the carious lesions

There was a high rate of incidence of carious lesions in the molars of the cotton rats fed the two basal synthetic rations 801 and 802 (table 3). The additional 6% casein contained in the latter ration did not decrease the rate of incidence nor the extent of the carious lesions in any way. Our groups of animals were small in all cases reported since only a limited number of animals was available because of our small stock colony. However, in every case the caries incidence and extent did not vary to any appreciable extent within any one group or between groups

when an experimental series was repeated. Therefore, the differences reported here were of importance.

The effect upon the incidence and extent of the carious lesions of various supplements to the synthetic rations is presented in table 3. It was observed that the addition of 4% 1:20 liver extract, alcohol ether extract equivalent to 6 or 9% of 1:20 liver extract, 4% solubilized liver or 4% whole liver substance to synthetic ration 801 did not alter the rate of incidence or the extent of carious lesions. When additional

TABLE 3

The effect of various supplements to synthetic rations upon the incidence of carious lesions in the cotton rat.

RATION	NUMBER OF WEEKS	NUMBER OF ANIMALS	AVERAGE NUMBER OF CARIOUS LESIONS (D)	AVERAGE EXTENT OF CARIOUS LESIONS (E)	AVERAGE EXTENT OF EACH CARIOUS LESION $\left(\frac{E}{D}\right)$
801	14	2	26.0	70.0 +	2.7 +
801 + 4% 1:20 L. E.	14	7	26.0	74.3 +	2.9 +
801 + alcohol ether extract equiv. to 6% 1:20 L. E.	14	2	25.5	64.5 +	2.6 +
801 + alcohol ether extract equiv. to 9% 1:20 L. E.	14	4	23.8	72.3 +	3.0 +
801 + 4% solubilized liver	14	2	29.5	73.5 +	2.5 +
801 + additional vitamins A, D, E, and K	14	4	20.3	36.5 +	1.3 +
801 + 4% whole liver substance	12	2	24.5	63.0 +	2.6 +
802	14	4	27.3	63.5 +	2.3 +
802 + 4% 1:20 L. E.	14	3	27.7	62.0 +	2.2 +
802 + alcohol extract equiv. to 6% 1:20 L. E.	14	3	27.0	63.3 +	2.3 +

amounts of the fat-soluble vitamins, A, D, E, and K were fed as supplements to ration 801, there was a slight reduction in the rate of incidence and a very substantial decrease in the rate of progress of decay in the lesions. Studies are now in progress to determine which of the fat-soluble vitamins was responsible for this decrease in rate and extent. This result may be similar to the effect attributed to vitamin K by Fosdick, Fancher and Calandra ('42). However, Armstrong, and Knutson ('43) and Armstrong, Spink and Kahnke ('43) explained the effect reported by Fosdick et al. as an effect due to quinones in general rather than any specific inhibiting action of vitamin K.

A supplement of 4% 1:20 liver extract or of an alcohol extract equivalent to 6% of 1:20 liver extract to ration 802 did not have any effect upon the rate of incidence or the extent of the carious lesions.

Both the Steenbock stock ration no. 14 and the ration in which dextrin was substituted for sucrose produced an almost complete prevention of carious lesions in the cotton rat molars (table 4). This was in contrast to the high incidence of carious lesions observed on the sucrose rations.

TABLE 4

The effect of coarse and fine rations upon the incidence of carious lesions in the cotton rat.

RATION	NUMBER OF WEEKS	NUMBER OF ANIMALS	AVERAGE NUMBER OF CARIOUS LESIONS (D)	AVERAGE EXTENT OF CARIOUS LESIONS (E)	AVERAGE EXTENT OF EACH CARIOUS LESION $\left(\frac{E}{D}\right)$
Coarse stock	12	3	6	12.0 +	2.0 +
Fine stock	12	3	6	8.0 +	1.3 +
Coarse dextrin	12	3	2	2.0 +	1.0 +
Fine dextrin	12	1	1	1.0 +	1.0 +
Coarse stock	50	23	3	7.6 +	2.5 +

The effect of particle size of the diet upon the incidence of carious lesions

The results obtained as a comparison of the effect of particle size on the incidence of carious lesions is presented in table 4. Difficulties were immediately encountered in the groups of animals fed either of the finely ground rations. The cotton rats almost completely refused to eat either of the fine rations. Instead they scattered it out of the food cup immediately after the daily feeding. Therefore, few of these animals survived the 14-week experimental period. In an effort to obtain as much data as possible, the surviving members were sacrificed at 12 weeks. It was found after that experimental period that no differences resulted in the rate of incidence of carious lesions when the particle size was varied. There was equal protection from decay in the molars on either the coarse or fine stock and dextrin rations.

DISCUSSION

The cotton rat is unquestionably more susceptible to tooth decay than any other experimental animal yet reported. The very high incidence of carious lesions in the molar teeth of the cotton rat raised on a high sucrose ration was in great contrast to the extremely low incidence observed when the sucrose of the ration was replaced by coarse or fine

dextrin. A similarly low incidence was observed in those animals fed either a coarse or fine stock ration. Since particle size did not appear to play any role in the development of the carious lesions in the molars of the cotton rat, this uncontrollable variable in the study of dental caries in the white rat was not encountered. Since the cotton rat is very susceptible to dental caries when fed certain rations, but extremely immune when on others, it can be used for many types of dental caries research. When such a wide difference in caries incidence is possible, the positive or negative value of the treatment given during the experimental period can be readily and accurately evaluated.

As yet only the supplement of additional vitamins A, D, E, and K, was found to have any preventative effect when a high level of sucrose was fed in the ration. However, this protective effect was much less than that observed when sucrose was replaced by dextrin or when a stock ration of common foodstuffs was fed. Studies are being conducted to determine which of these vitamins was responsible and if the effect was due to that particular vitamin or to the class of compounds to which it belongs.

There was an almost complete bilaterally equal distribution of the carious lesions in both the upper and lower jaws. This has been shown to exist in man by Bertram and Brown ('43).

The upper jaw has a very much lower incidence of carious lesions than the lower jaw. This has been reported by Klein and Palmer ('41) for the first and second permanent molars of the human.

SUMMARY

1. The cotton rat is highly susceptible to tooth decay when fed a synthetic ration high in sucrose.

2. Almost no tooth decay occurred in those cotton rats which were fed a stock ration composed of natural foodstuffs or a synthetic ration in which the sucrose had been replaced by dextrin.

3. An experimental period of 14 weeks was found to be optimal for the observation and evaluation of the carious lesions.

4. No differences in the rate of incidence or the extent of the carious lesions could be produced by altering the particle size of the stock or dextrin rations.

5. The various liver supplements which were added to the sucrose rations to produce optimal growth had no effect on the development of the carious lesions.

6. Increased amounts of the fat-soluble vitamins, A, D, E, and K, produced a decrease in the number and the extent of the carious lesions in the molars of cotton rats on the sucrose ration 801.

7. There was a definite bilateral distribution of the carious lesions. There was a much lower rate of incidence of carious lesions in the molars of the upper jaw than in those of the lower jaw. The ascending order of caries incidence in the molars was as follows: upper first, lower first, upper third, upper second, lower second and lower third.

8. In view of these observations the authors believe that the cotton rat is the best experimental animal yet known for the production and study of tooth decay. The ease of production and the high, consistent incidence of carious lesions make possible many phases of experimental approach to the study of tooth decay, its causes and its control.

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THE RELATION OF THE BLOOD LEVEL OF ASCORBIC ACID TO THE TISSUE CONCENTRATIONS OF THIS VITAMIN AND TO THE HISTOLOGY OF THE INCISOR TEETH IN THE GUINEA PIG [1]

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FIVE FIGURES

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Information concerning the relation of the blood level of vitamin C to the well-being of the individual is of considerable interest. Such information should involve the relation of the blood level to the concentration of the vitamin in the other tissues of the body and to the presence or absence of any pathological changes. With such knowledge one could make an appraisal of the nutritional status, with respect to vitamin C, of an animal or of a human subject, by determining the vitamin C content of the blood. In this paper, we are reporting the results of a study of these relationships in guinea pigs upon different levels of intake of vitamin C.

EXPERIMENTAL PROCEDURE

Sixty-six guinea pigs, thirty-three males and thirty-three females, of market stock with initial weights ranging from 188 to 517 gm. were maintained for 10 days on rabbit chow [3] plus cabbage ad libitum. This was to bring their body stores of vitamin C to approximately the same level. At this time they were transferred to a basal diet composed of:

Ground rolled oats (Quaker)	40 parts
Lactogen [4]	40 parts
Alfalfa meal	14 parts
Dried irradiated brewer's yeast [5]	5 parts
Sodium chloride	1 part
	100 parts

[1] Some of the data reported in this paper are taken from a dissertation submitted by Carl A. Kuether to the Graduate Council, George Washington University, April 1943, in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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[3] Maritime Milling Co.

[4] Spray dried whole milk modified by the addition of milk fat and milk sugar. We wish to thank Nestle's Milk Products, Inc., for a generous supply of Lactogen.

[5] Fleischmann, Std. 360.

Supplemented with ascorbic acid, this diet produced good growth and prevented any obvious signs of deficiency in the guinea pig (Roe, Hall and Dyer, '41). Analysis showed this diet to contain 28 mg. of ascorbic acid per kilogram. The animals were fed this diet ad libitum after the following amounts of crystalline ascorbic acid [6] had been added per kilo of diet: 0, 50, 100, 150, 200, 300, 400, and 600 mg. A group of animals was placed on each level of ascorbic acid and one group was fed approximately 50 gm. of cabbage daily in addition to the basal diet. The animals were kept individually in cages with raised wire screen bottoms. Food consumption and weights were recorded daily. Ascorbic acid intakes were calculated from the food intakes.

Careful consideration was given to the problem of placing the animals on the test diets for a period that would produce a fairly constant level of ascorbic acid in the tissues for a significant time. Preliminary experiments showed that the tissue ascorbic acid content remained practically constant after the animals had been on a diet of the same ascorbic acid concentration for a period of 3 weeks. For example, the vitamin C concentration in the livers of guinea pigs taken off the preparatory diet and maintained at a constant low intake of vitamin C were 5.48, 4.24, 4.18 and 4.21 mg. per 100 gm. at 2, 3, 6, and 7 weeks, respectively, and the corresponding concentrations in the spleen were 17.7, 10.7, 11.5 and 10.4 mg. per 100 gm. It was decided to follow the more normal feeding procedure of incorporating the vitamin in the diet, so the animal would receive the supplement no faster than food is taken, rather than to give it in peak loads as when administered in solution by pipette or stomach tube.

After the animals had been on the diet for appropriate periods they were anaesthetized by an intraperitoneal injection of approximately 30 mg. of Nembutal per kilogram of body weight. A blood sample was collected by heart puncture and the tissues were rapidly removed for analysis. The tissues analyzed were: whole blood, liver, spleen, kidney, adrenal glands, skeletal muscle (gastrocnemius), cardiac muscle and brain. No animal was sacrificed until after having been on the test diet for at least 21 days. The average time for the groups of animals upon each of the test diets ranged from 26 to 38 days except one group, no. 8 of table 1, which was kept on the same level of vitamin intake for 73 days. The concentrations of ascorbic acid in the tissues of the latter group fall quite consistently in line with the levels in the tissues of the animals in the 26- to 38-day test periods. This similarity of results of analyses in the very long test period is further evidence that the 26- to

[6] We wish to thank Merck and Co. for supplying the ascorbic acid.

38-day periods were long enough to produce characteristic physiological or pathological findings in the animals.

Whole blood analyses were made by the method of Roe and Kuether ('43). Tissue analyses were made on a filtrate prepared by grinding the tissue to a uniform suspension with sand and 4% trichloroacetic acid. This was treated with Norit and filtered through a dry filter. The filtrate was analyzed as described by Roe and Kuether for blood filtrates.

The carcasses were examined grossly for the presence of hemorrhage, particularly around the knee joints and in the stomach, enlargement of the joints and beading of the costochondral junctions. The jawbones were removed and preserved in 10% neutral formalin for sectioning and microscopic examination of the incisor teeth.

RESULTS

Chemical findings

The results of the tissue analyses are summarized in table 1. Ascorbic acid intakes were calculated from the mean daily food consumption and body weight for the 14 days immediately preceding analysis. Increasing the level of ascorbic acid intake increased the ascorbic acid concentration in the blood and all the tissues analyzed, although not to the same extent. In going from the lowest to the highest intake the concentration of ascorbic acid in the whole blood increased about 6 times, the corresponding increases for the other tissues were: brain 6, muscle 11, kidney 14, spleen 15, heart and liver 19 and the adrenals 24 times. Thus there is considerable variation in the ability of the tissues studied to store vitamin C. From the values for the heart and skeletal muscle it is apparent that the concentration in cardiac muscle averages approximately 2½ times the concentration in skeletal muscle. Histologically and functionally cardiac muscle differs from skeletal muscle and it is not surprising to find this difference in ascorbic acid content.

It is questionable whether the tissue concentrations reported here represent the maximum concentrations attainable since higher values than these have been reported. Roe, Hall and Dyer ('41) published figures showing tissue concentrations per 100 gm. to be as high as the following: liver, 37 mg.; spleen, 42 mg.; brain, 24.5 mg.; and adrenal gland, 336 mg. These values were found in guinea pigs fed rabbit chow and cabbage. The maximum values found in the present study were: liver, 32.8; spleen, 49.1; kidney, 11.6; adrenal, 166; skeletal muscle, 3.12; brain, 22.8; heart, 8.98; and whole blood, 1.16 mg.%. With higher intakes of ascorbic acid it appears possible to raise the tissue concentrations to higher levels. In the group of animals with the highest meas-

TABLE 1

Tissue ascorbic acid concentrations in guinea pigs upon different ascorbic acid intakes. All tissue concentrations are expressed in milligram per cent.

GROUP	INTAKE [1]	NO. ANIMALS	DAYS [2] ON DIET	WHOLE BLOOD	LIVER	SPLEEN	KIDNEY	ADRENAL	MUSCLE	BRAIN	HEART
1	.179 ± .027 [3]	9	26	.088 ± 0.19	.79 ± .13	2.25 ± .52	.55 ± .13	4.52 ± .90	.20 ± .03	3.05 ± 1.04	.29 ± .04
2	.532 ± .047	8	32	.139 ± .094	2.46 ± 1.03	7.98 ± 3.09	1.65 ± .68	16.78 ± 7.88	.52 ± .26	5.55 ± 1.57	1.09 ± .48
3	.701 ± .033	5	38	.143 ± .032	3.75 ± 1.13	12.76 ± 2.75	2.48 ± .43	28.52 ± 6.55	.76 ± .14	8.35 ± 1.95	2.16 ± .24
4	.900 ± .062	6	26	.193 ± .019	4.93 ± 1.04	17.05 ± 2.75	3.10 ± .46	37.73 ± 11.36	.86 ± .14	10.47 ± 2.77	2.56 ± .37
5	1.23 ± .12	7	31	.256 ± .043	7.68 ± 1.66	20.88 ± 3.16	4.35 ± .62	53.2 ± 10.6	1.13 ± .16	13.2 ± 1.5	3.35 ± .61
6	1.53 ± .09	7	35	.276 ± .065	9.71 ± 2.87	25.19 ± 4.60	5.14 ± 1.03	59.3 ± 15.0	1.34 ± .42	14.6 ± 2.1	3.83 ± .75
7	1.83 ± .08	7	34	.355 ± .082	11.59 ± 3.55	25.99 ± 4.40	5.64 ± 1.50	73.9 ± 15.8	1.63 ± .27	15.2 ± 2.2	4.59 ± .90
8	3.44 ± .72	9	73	.538 ± .192	15.45 ± 3.94	33.05 ± 6.86	7.87 ± 1.83	110.5 ± 27.9	2.24 ± .30	18.4 ± 3.0	5.49 ± .16
9	Cabbage	8	37	.754 ± .212	23.77 ± 6.02	42.83 ± 1.79	9.14 ± 1.68	118.6 ± 26.3	2.39 ± .42	18.6 ± 1.3	7.54 ± 1.29

[1] Milligram of ascorbic acid per 100 gm. body weight per day.

[2] Number of days the animals were on this intake before analysis (mean).

[3] Standard deviation of mean.

ured intake, namely 3.44 mg. per 100 gm. of body weight per day, the tissues had not yet reached saturation, since the group receiving cabbage had higher levels.

The ratios of the tissue concentrations of ascorbic acid to the whole blood concentration are shown in figure 1. With each tissue the ratio increases with increasing whole blood concentration to a maximum value. Increasing the whole blood concentration beyond this point resulted in a decrease in the ratio for spleen, kidney, brain, adrenal gland, heart and skeletal muscle, and no further increase in liver. The increasing ratio shows that the tissue concentrations of ascorbic acid are increasing at a faster rate than the whole blood concentration, indicating an active storage of vitamin C by the tissues. In every tissue studied the ratio reaches a maximum value at, or near, a whole blood concentration of 0.25 mg.%. This finding means that the tissues withdraw ascorbic acid from the blood most efficiently when the blood level reaches a concentration of 0.25 mg. per 100 ml. of whole blood. A higher blood level would have an advantage, however, in that it would bring about a greater storage of the vitamin for an emergency. The importance of these data is that they show the whole blood level of ascorbic acid reliably reflects the concentration of the vitamin in the tissues, hence a whole blood determination of ascorbic acid may be accepted as a significant indication of the nutritional status of the animal with respect to this vitamin.

From table 1, it will be seen that to maintain a whole blood concentration of 0.25 mg. per 100 ml., an intake of approximately 1.23 mg. of ascorbic acid per 100 gm. of body weight per day is necessary. If this rate of intake were transferred to man on the basis of body weight, the intake of a 70 kg. man would be 861 mg. per day. Translating upon a basis of body surface, the corresponding intake for man is calculated as follows. Using Meeh's formula for calculating surface area (Meeh, 1879; Lusk, '28, p. 123), the surface area of a 400 gm. guinea pig is 4.61 sq. dm. This makes the guinea pig requirement 1.07 mg. per sq. dm. per day when the whole blood level is maintained at 0.25 mg. per 100 ml. For an average man, height 5 ft. 8 in., weight 70 kg., the surface area (DuBois and DuBois, '16) is 1.83 sq. m. or 183 sq. dm. An intake equivalent to 1.07 mg. per sq. dm. per day would be 196 mg. for a man of 183 sq. dm. surface. Thus these studies indicate, by either a body weight or surface area translation of data, that the vitamin C requirement of the guinea pig is much greater than the requirement of man.

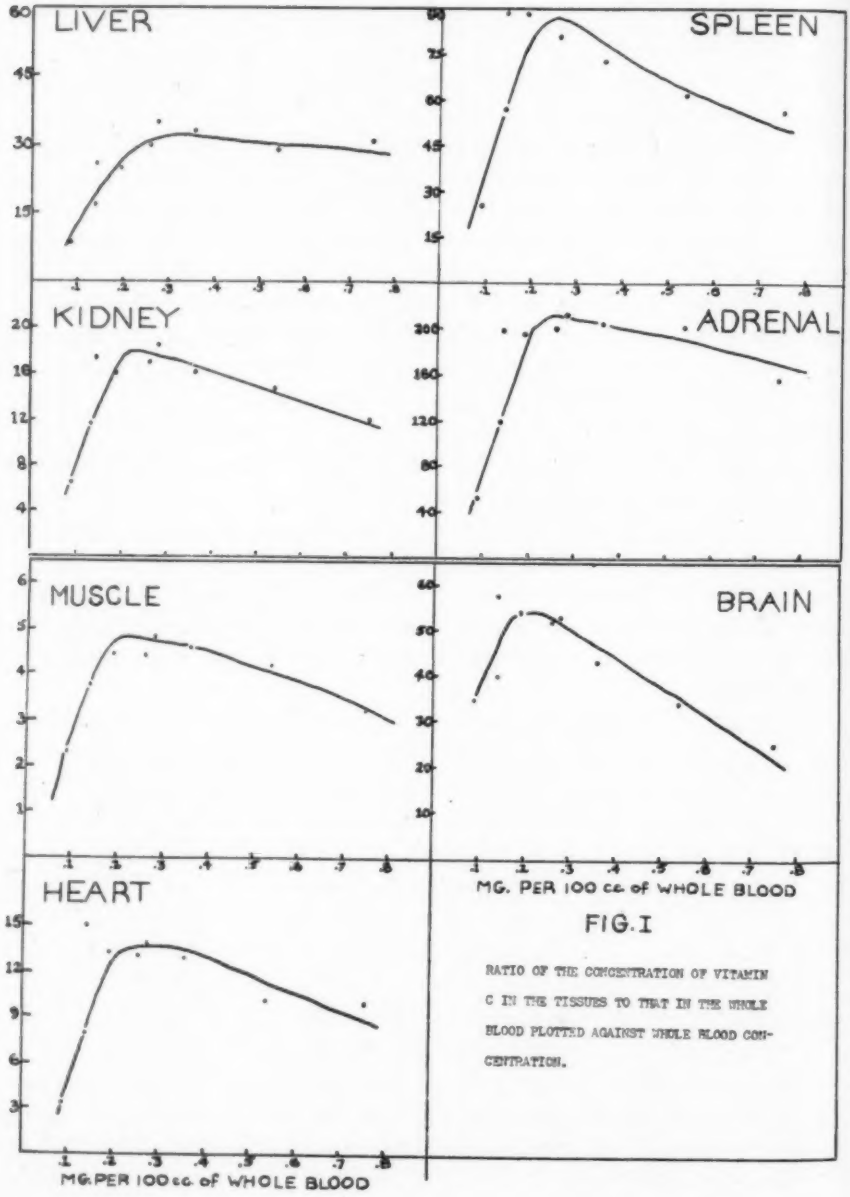

FIG. I

RATIO OF THE CONCENTRATION OF VITAMIN C IN THE TISSUES TO THAT IN THE WHOLE BLOOD PLOTTED AGAINST WHOLE BLOOD CONCENTRATION.

Histological findings

The observations of Zilva and Wells ('19) revealed that the teeth were the first of the body tissues of the guinea pig to show an abnormality during the development of scurvy. Other histological work on the lesions in teeth of scorbutic guinea pigs has been done by Toverud ('23), Höjer and Westin ('25), Goettsch and Key ('28), Key and Elphich ('31), Fish and Harris ('34) and Boyle and his associates ('38, '40).

For the histological studies, fourteen male and twelve female guinea pigs were selected from the nine groups mentioned above. Each mandible with all teeth intact was fixed in 10% neutral formalin, decalcified in an alcoholic 5% HNO_3 solution, embedded in nitrocellulose, sectioned longitudinally and stained with hematoxylin and eosin, and Mallory's triple connective tissue stain. These sections were then carefully studied for the presence of any lesions resulting from the dietary regimen of each group. The specific areas subjected to critical study were predentine layer, dentine with odontoblastic layer, enamel with ameloblastic layer, pulp cavity, cementum, periodontal membrane and the alveolar bone.

The use of longitudinal sections of the incisor teeth instead of cross sections, as are commonly used (Höjer and Westin, '25; Höjer, '26; Goettsch and Key, '28; Key and Elphich, '31; Dann and Cowgill, '35), has several advantages. Attention is called to the fact that each cross sectional level of the continually growing incisor tooth of the guinea pig has a different histological picture. The odontoblasts and ameloblasts alter their cytological structure and show senile changes as they migrate to the incisoral end of the tooth. Thus cross sections at different levels have a different histological appearance. Any comparisons of such cross sections could, therefore, lead to erroneous conclusions. Furthermore, longitudinal sections record the entire history of any recent acute scurvy. Changes in the incisor teeth, occurring early in the course of the deficiency, will still be observable at a later date in the sensitive cell layers which have subsequently migrated nearer the incisoral end of the tooth.

We studied incisor teeth rather than molar teeth (Fish and Harris, '34) because in the guinea pig the incisors grow, erupt and calcify continually throughout life. They show, therefore, in their longitudinal sections, the complete cycle of an acute ascorbic acid deficiency. The molar teeth were of little value in our experiments because their growth is largely limited to the early periods of life and hence are not sensitive

to the recent acute manifestations of the disease. Since the dentine and enamel layers in the molars of the guinea pig are of limited growth, the critical cell layers, the odontoblasts and ameloblasts, are largely dormant, inactive, senile cells and not sensitive indicators of mild scorbutic conditions.

There are two separate pathological changes occurring in the incisor teeth of guinea pigs upon a diet deficient in ascorbic acid. The first change is an upset in the calcification processes of the tooth. This is seen in several of the layers. The predentine layer becomes calcified. Normally this layer is comparatively free of calcium. The dentine often shows a mottling effect accompanied by areas or zones of decalcification. The pulp cavity shows spotty areas of irregular foci of calcification. The bone sections show some decalcification or faulty calcification of the alveolar bone. The cementum in animals on low vitamin C intake is largely replaced by a calcified matrix with entrapped cementoblasts.

The second constant change is a degeneration in the parenchymal cells of the teeth. The odontoblasts are the most sensitive of the parenchymal cells to a vitamin C deficiency (figs. 3, 4 and 5). In early ascorbic acid deficiency these cells become disorganized, vacuolated and show early signs of degeneration (fig. 3). With ascorbic acid blood levels below 0.15 mg. per 100 ml. these cells are completely degenerated (figs. 4 and 5). The ameloblasts are less sensitive to the lower levels of ascorbic acid intake (fig. 4) and show degeneration only in the most severe scurvy. The other cells — the cementoblasts, osteoblasts, and fibroblasts — show premature atrophy, loss of normal function and general disintegration.

These studies show clearly that the tooth is a good biological indicator of the nutritional status of the guinea pig in respect to vitamin C. The use of the teeth in guinea pigs is a much more accurate and sensitive means of detecting early scurvy symptoms than the usual methods. The presence of hemorrhages, especially around the knee joint, has been widely used as an indicator of scurvy. The appearance of such hemorrhages, however, is a gross manifestation of a severe deficiency and is of no value in the detection of mild scurvy. Our results show that tooth lesions appear at much higher blood levels of ascorbic acid than do the signs of hemorrhages (table 2). The retardation of growth has long been used as an indicator of ascorbic acid deficiency. Table 2 shows that growth still occurred in animals held for 4 to 5 weeks on a diet low in ascorbic acid. The odontoblasts, however, indicated varying degrees of the deficiency which were not shown by growth observations. Thus, the incisor teeth of the guinea pig, and more specifically the

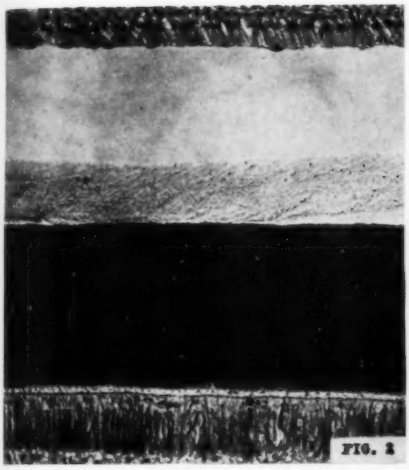
FIG. 2

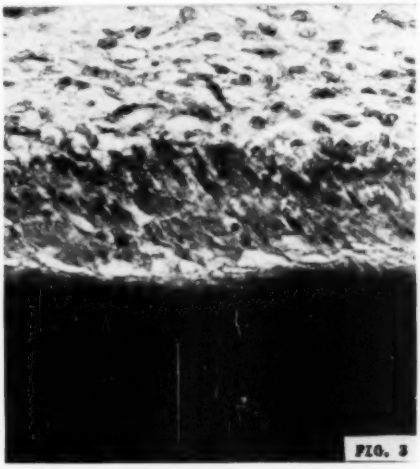
FIG. 3

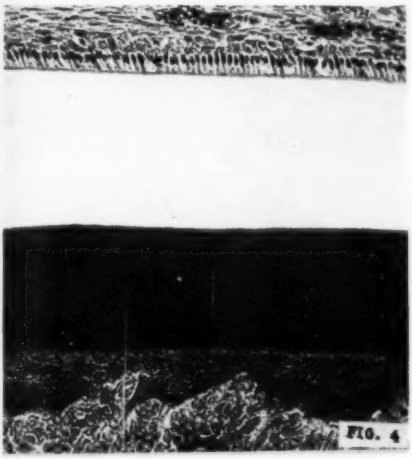
FIG. 4

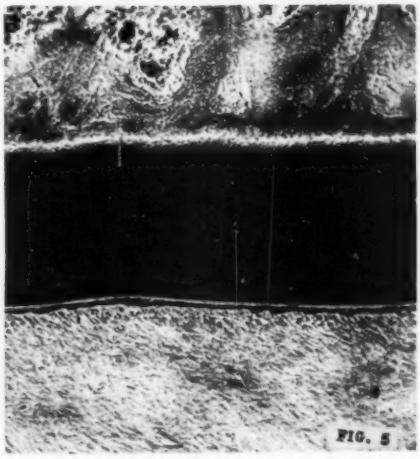
FIG. 5

Fig. 2 Guinea pig no. 163 — 0.42 mg. of ascorbic acid per 100 ml. of blood. Shows normal dentine, odontoblasts, predentine, enamel space and ameloblasts. Mag. 146 ×.

Fig. 3 Guinea pig no. 148 — 0.15 mg. of ascorbic acid per 100 ml. of blood. Shows disorganization of odontoblastic layer, dilation and confluence of Tome's canals. Mag. 305 ×.

Fig. 4 Guinea pig no. 144 — 0.08 mg. of ascorbic acid per 100 ml. of blood. Shows persistence of ameloblasts but disappearance of odontoblasts. Note the narrowing of the dentine layer. Mag. 146 ×.

Fig. 5 Guinea pig no. 144 — 0.08 mg. of ascorbic acid per 100 ml. of blood. Shows degeneration of odontoblasts and disorganization of periodontal membrane. Mag. 146 ×.

parenchymal cells of these teeth, offer a sensitive measure of ascorbic acid deficiency.

The present studies confirm the observations of other investigators (Zilva and Wells, '19; Toverud, '23; Höjer and Westin, '25; Key and Elphich, '31; Dann and Cowgill, '35; Boyle, Bessey and Howe, '40),

TABLE 2

The relation of the blood level of ascorbic acid to the nutritional state of the guinea pig.

GUINEA PIG NUMBER AND SEX		MG. ASCORBIC ACID PER 100 ML. OF WHOLE BLOOD	DEGREE OF TOOTH PATHOLOGY	HEMORRHAGE IN KNEE JOINT	WEIGHT GAIN GM./DAY [1]
142	F	0.06	+ + + +	Marked	3.1
132	F	0.07	+ + + +	Marked	1.5
144	M	0.08	+ + + +	Slight	0.0
137	F	0.12	+ + +	Slight	— 2.1
173	M	0.14	+ + +	Slight	4.7
148	F	0.15	+ +	None	2.6
151	M	0.15	+ +	None	3.9
171	M	0.16	+ + + +	None	6.4
183	M	0.18	+	Slight	5.1
174	F	0.19	+ +	None	5.8
185	M	0.19	+ +	None	4.0
184	F	0.20	+	None	5.6
191	M	0.21	+	None	0.4
199	M	0.22	Normal	None	3.7
187	M	0.22	Normal	None	2.3
201	M	0.28	Normal	None	2.9
156	F	0.28	Normal	None	0.1
200	F	0.32	Normal	None	3.8
206	F	0.34	Normal	None	2.4
202	F	0.37	Normal	None	4.4
163	M	0.42	Normal	None	0.3
161	M	0.43	Normal	None	3.7
162	F	0.46	Normal	None	2.3
165	M	0.73	Normal	None	3.4
124 [2]	F	0.77	+	None	— 0.9
167	M	1.16	Normal	None	5.1

[1] For last 14 days.

[2] This animal was sick, refused food and lost weight.

which have shown that, of the various tissues of the body of the guinea pig, the incisor teeth are the most sensitive to a vitamin C deficiency. In addition we have established the blood and tissue ascorbic acid concentrations which result in a normal histological picture, and the levels at which pathological lesions occur. In table 2, a whole blood concentration of 0.22 mg. of ascorbic acid per 100 ml. represents a definite border-line where the earliest pathological signs of a deficiency occur

in the incisor teeth of guinea pigs. This level was determined by a histological diagnosis taking into consideration all of the criteria discussed above. The diagnoses were made by one of us (I.R.T.) without knowledge of the blood concentrations of ascorbic acid. In view of the rather narrow range of blood concentrations (0.08 mg. to 0.22 mg. of ascorbic acid per 100 ml.) above which normal histology was observed and below which the most severe pathological changes were found, table 2 shows a striking correlation between chemical findings and histological observations.

SUMMARY

1. A study of the relation of the blood concentration of ascorbic acid to the tissue concentrations and to the histology of the teeth has been made in the guinea pig.

2. The most efficient rate of withdrawal of ascorbic acid from the blood by liver, spleen, kidney, adrenal gland, brain, skeletal muscle and cardiac muscle occurs at a whole blood concentration of this vitamin near 0.25 mg. per 100 ml.

3. A concentration of ascorbic acid in the tissues reflected by a blood level above 0.22 mg. per 100 ml. of whole blood will prevent the appearance of any pathological lesions in the incisor teeth of the guinea pig.

4. The most obvious pathological changes due to ascorbic acid deficiency are to be found in the odontoblastic layer.

5. An alteration in the calcification pattern of the incisor teeth occurs at about the same blood levels as those associated with beginning changes in the odontoblastic layer, but the former changes are not as consistent or as easily interpreted.

6. Conventional methods of determining the presence of scurvy, such as the appearance of hemorrhages, reduced growth rate, or loosening of the teeth, are too inaccurate and insensitive to be of any value in detecting a mild ascorbic acid deficiency in the guinea pig.

7. An intake of 1.23 mg. of ascorbic acid per 100 gm. of body weight is required in the guinea pig to produce a whole blood level of 0.25 mg. per 100 ml. Translated to man, this requirement would be equivalent to an intake of 861 mg. per day on a basis of body weight or 196 mg. per day on a surface area basis. These data indicate that the vitamin C requirement of the guinea pig is higher than that of man.

8. Since there has been observed in the guinea pig a definite relation between the concentration of ascorbic acid in the tissues and that in the blood, it is suggested that the determination of the ascorbic acid content of the whole blood is the best procedure for evaluating the nutritional status of an animal with respect to vitamin C.

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THE EFFECT OF FEED ON THE CRITICAL TEMPERATURE FOR THE ALBINO RAT [1]

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ONE FIGURE

(Received for publication June 15, 1944)

Results reported in a previous publication on this subject (Black and Swift, '43) failed to verify the prevailing understanding that the critical temperature for an animal receiving food is lower than for the same animal during fasting. In this study the curve of heat production in relation to the environmental temperature for rats receiving feed was found to be similar to the one obtained earlier (Swift and Forbes, '39) for fasting rats except that somewhat more heat was produced in the feeding periods at temperatures above 26°C. The authors, however, were careful to point out that failure to find any effect of food on the critical temperature did not prove that such an effect is non-existent.

The problem has recently been subjected to further investigation with three improvements in technique, namely; (1) the heat production of the same six rats was determined during feeding and during fast, whereas in the previous studies different groups of rats were used for these two conditions; (2) the experimental subjects were reared at a practically constant environmental temperature of 30°C., which was within the zone of thermal neutrality, whereas in the earlier work they were reared at a slightly subcritical temperature; and (3) the diet fed was devised in the light of results obtained in other experiments at this laboratory (Forbes and Swift, '44) in a manner calculated to produce a maximum dynamic effect.

Six mature, male, albino rats were used in this study, which extended over a period of 3½ months. The ration consisted of a mixture of the stock colony diet [2] (56.2%) and ether-extracted beef muscle (43.8%). The ration contained 50.1% protein, and was characterized by an energy value of 4558 cal. per gram. The rats were fed nearly the maximum

[1] Authorized for publication on June 13, 1944 as paper no. 1238 in the Journal Series of the Pennsylvania Agricultural Experiment Station.

[2] Purina dog chow.

quantity they would consume, five of them each eating 14 gm. of feed per day, and the sixth animal consuming 11 gm. The daily allotment of feed was weighed out in two equal portions and fed at 7 A.M. and 5 P.M. The feed intake was constant throughout the entire experiment, except that preceding the fasting periods, which were always at least 8 days apart, two consecutive portions of feed were withheld. Thus, a rat entering the respiration chamber at 8 A.M., in a fasting experiment, would have consumed its most recent portion of feed, about 24 hours earlier.

The apparatus and procedure were similar to those which have been described in previous publications (Swift and Forbes, '39; Black and Swift, '43). Respiration measurements began at about 8 A.M. and continued for 8 hours. During this interval the CO_2 was determined at the end of the first, second, third, sixth, and eighth hours. The entire period was used to determine the respiratory quotient and total oxygen consumption, while for the computation of the metabolism during rest, the subperiods of inappreciable activity and uniform CO_2 production were used.

A distinct advantage of using the same six rats throughout the entire experiment was that, as the animals became accustomed to the routine, they remained quiet, not only while being weighed on a large chainomatic balance at the beginning and end of each respiration period, but also during a large part of the 8 hours spent in the respiration chamber. This is important, since the selection of the intervals of CO_2 measurement to represent the metabolism during rest is fraught with some degree of uncertainty even when work adders are used as indicators of activity. In order to make such selection less arbitrary, in the experiment here reported, the intervals used to represent the metabolism during minimum activity were those in which the CO_2 fell within $\pm$ 6% of the average for the day for the animal in question, excluding from the average a preliminary period since the animal was not exposed to the temperature of the respiration chamber until the beginning of this period. Furthermore, it is to be expected that there would be a lag in the physiological response of the animal to the environmental temperature.

The temperature of the respiration chamber, immersed in a water bath, was higher than that of the bath, especially at the lower temperatures. At a bath temperature of 12.9°C. the chamber registered 15°C., whereas at a bath temperature of 31°C., or above, the difference was negligible. The temperature inside the chamber was taken as the environmental temperature.

After the respiration measurements were completed, the urine and feces were collected separately during a feeding period of 8 days. The urinary nitrogen was used as a basis for the derivation of the heat produced by the oxidation of protein, and for the derivation of the non-protein respiratory quotient in the usual manner. In the fasting periods, the respiratory quotients were not corrected for the protein metabolism.

The chronological order in which the experiments were conducted is shown in table 1. In all cases, any feeding period was followed immediately by a fasting period at the same temperature. The live weight of the rats during the 3½ months was nearly constant, increasing from an average of 326 gm. to 336 gm. for the feeding periods, and from 300 gm. to 311 gm. during the fasting periods. The fasting heat production of each rat for each day was computed to a live weight of 310 gm. in accord with the 0.73 power of the live weight. The heat production of the feeding periods was used as determined.

The use of the same rats throughout the experiment allowed the application of Student's method in evaluating the results, the measurements of heat production as obtained from a given rat at two different temperatures constituting a pair of observations. The odds of significance shown in table 1 have been derived by comparing the values for the heat production of each of the six rats with the corresponding values at different temperatures. It is obvious that if the differences in environmental temperature are very small, no statistically significant difference could be shown to exist between the values for heat production at any two consecutive temperatures. The temperatures for which the heat productions were compared in evaluating the significance of the differences between these values, are indicated in table 1.

The respiratory quotients in all feeding and fasting periods were extremely uniform among the 6 rats for any given temperature. In accord with a finding by Swift ('32) that protein metabolism is unaffected by exposure to cold, it was considered that the protein metabolism in the feeding periods, at all temperatures, was represented without significant error by the urinary nitrogen obtained during the 8-day collection period, in which the environmental temperature was very close to 30°C. It is interesting to note that, throughout the range of thermal neutrality of the environment, about 71% of the total heat was derived from protein, while at 15°C. protein furnished 41% of the heat, with a concomitant increase in the amount of fat oxidized.

When this study was undertaken it was considered that the effect of feeding might be not only to lower the critical temperature, but that with fall in the temperature below the critical for fasting there would

TABLE 1

Heat production of fed and fasting rats with change in environmental temperature.

ORDER OF EXPERIMENTS	TEMPERATURE	FEEDING PERIODS					FASTING PERIODS				
		Avg. live weight	Avg. R.Q.	Avg. heat per hour	Odds of significance		Avg. live weight	Avg. R.Q.	Avg. heat per hour	Odds of significance	
					With reference to preceding temperature	With reference to second preceding temperature				With reference to preceding temperature	With reference to second preceding temperature
	°C.	gm.		cal.			gm.		cal.		
9	15	340	.85	2501	....	....	318	.72	2392	....	....
8	23	340	.90	1677	10,000 to 1	....	318	.74	1523	10,000 to 1	....
7	26	342	.91	1531	275 to 1	10,000 to 1	315	.73	1233	3,332 to 1	10,000 to 1
6	27	335	.91	1434	26 to 1	481 to 1	314	.74	1161	19 to 1	5,000 to 1
5	28	329	.91	1410	6 to 1	57 to 1	310	.74	1066	243 to 1	908 to 1
4	29	327	.92	1395	4 to 1	130 to 1	304	.74	1047	3 to 1	243 to 1
1	30	326	.91	1400	2 to 1	2 to 1	300	.74	1083	12 to 1	3 to 1
3	31	328	.92	1431	8 to 1	10 to 1	304	.76	1058	9 to 1	3 to 1
2	32	323	.93	1477	7 to 1	118 to 1	299	.75	1069	2 to 1	3 to 1
10	33	336	.93	1573	302 to 1	275 to 1	315	.77	1092	6 to 1	9 to 1
11	34	...	..	....	....	....	311	.78	1187	45 to 1	454 to 1

be no increase in heat production during feeding periods until all of the heat increment of the food as observed during thermal neutrality had been utilized to prevent an increased metabolism due to cold. However, figure 1 shows that only about two thirds of the heat increment was effective in this respect, since the difference between the heat production of feed and of fast was about one third as great at subcritical temperatures as within the zone of thermal neutrality. In other words, we may consider that at subcritical temperatures, at least from 15° to 23°C., about two thirds of the heat increment is "useful" to the rat. It is recognized that any effect which feeding or fasting may have exerted on the constancy of body temperature is an integral part of the results obtained.

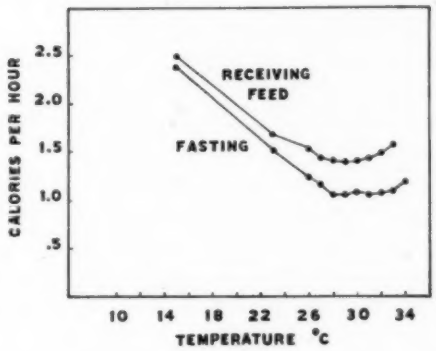

Figure 1

The results depicted in figure 1, and in table 1, are largely self-explanatory. The range of thermal neutrality for fasting rats is shown to be from 28° to 33°C. The observed critical temperature (28°C.) is 2 degrees lower than as reported in a previous publication (Swift and Forbes, '39), the upper limit of the zone of thermal neutrality being the same as previously found. It is possible that the residual effect of the very high protein diet fed in the present experimental work accounts for this difference. The range of thermal neutrality for the rats while receiving feed was from 27° to 32°C. Thus, the consumption of feed characterized by a high dynamic effect resulted in the lowering of both the upper and the lower limits of the zone of thermal neutrality by 1°C.

It is concluded, therefore, that energy metabolism studies with albino rats, either with or without feed, may properly be conducted at 30°C.

The plotting of the data for each rat produced a curve of heat production closely similar to the curve representing the six animals as shown in figure 1.

At the start of the experiment five of the six rats were 6 months of age, the sixth animal being slightly more than 1 year old. Each rat was fasted a total of eleven times, and in ten instances the oldest individual produced more heat (correlated to uniform live weight) at each temperature than did any of the younger rats. At all temperatures the heat production of the oldest rat exceeded the average heat production for the six rats. The amount by which the heat production of the oldest rat exceeded the average heat production of the other five rats in the eleven fasting periods was 12.9%. This is in accord with a result reported by Benedict and MacLeod ('29) who found that the metabolism of fasting rats increases with age.

SUMMARY

The heat production of six mature, male, albino rats was determined while receiving a diet characterized by a high dynamic effect, and also while fasting, at environmental temperatures ranging from 15° to 34°C. The zone of thermal neutrality for the fasting rats was found to be from 28° to 33°C., the effect of feed being to lower both limits of this range by 1°C. About one third of the heat increment of the food as observed in the zone of thermal neutrality was manifest below the critical temperature.

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THE BIOLOGICAL ASSAY OF VITAMIN A BY MEANS OF ITS INFLUENCE ON THE CELLULAR CONTENTS OF THE VAGINA OF RATS

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ONE FIGURE

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Evans and Bishop ('22) observed that young rats fed a vitamin A deficient diet failed to show the normal cyclic changes in the cellular contents of the vagina and the squamous cells normally present during estrus were seen continuously. Attempts were made by a number of workers, Coward ('29), Hohlweg and Dohrn ('30), Bauman and Steenbock ('32), Moll et al. ('33), Coward et al. ('35), Coward ('38) and Goss and Guilbert ('39) to use this phenomenon as a criterion of response for the biological assay of vitamin A and its precursors.

In view of the relatively low degree of precision attained, duration of the test and the difficulties encountered in performing a satisfactory assay by the methods outlined in the United States Pharmacopoeia XII ('42) and British Pharmacopoeia ('32) addendum ('36) it appeared worthwhile to make a further study of the vaginal smear method to see if a practical and convenient procedure for the biological assay of vitamin A could be obtained yielding more precise values than those depending on an increase in body weight.

METHOD

During a period of 10 to 12 days 100 or more female rats at 21 days of age are obtained from the stock colony. They are fed the maintenance diet (table 1) and kept in screened-bottom cages until vaginal introitus occurs in the whole group.[1] At this time the whole group is ovariectomized (Burn, '37), and 2 to 3 days following the operation vaginal smears are taken from the whole group. Depending on the dominant type of cell present in the smear the rats are retained on the maintenance diet or fed the vitamin A free diet (table 1). If leucocytes

[1] The mean age of vaginal introitus in the Laboratory of Hygiene's colony of rats of Wistar strain is 45.2 ± 4.1 days.

predominate, the rats are fed the vitamin A free diet and the smears are examined bi-weekly until there is a dominance of squamous cells mixed with epithelial cells and leucocytes (stage 3, see below). Some of the rats reach this stage or one approaching it on the maintenance diet and they are retained on this diet until the remainder of the group shows the same evidence of depletion. When the whole group is near depletion, as shown by the vaginal smear, the rats are weighed, placed on the vitamin A free diet and smears are examined daily until all show uniformly a dominance of squamous cells (stages 1 and 2). This usually requires 3 to 4 days. The rats are weighed again and any showing a marked loss in weight (10 gm. or more) are excluded. The rats are divided into at least six groups on the basis of equal distribution of body weight. Three of the groups receive doses of the standard and

TABLE 1

Diets used for the biological assay of vitamin A by the vaginal smear technique.

COMPONENT	DIET	
	Vitamin A free	Maintenance
Rice starch, dextrinized	65%	58%
Casein, vitamin-free (Labco)	18%	18%
Brewers' yeast, fat-free	8%	..
Brewers' yeast	..	8%
Corn oil (Mazola)	5%	5%
Wheat germ	..	7%
Salt mixture (U.S.P.XII)	4%	4%
Vitamin D (viosterol): per 100 gm.	300 I.U.	300 I.U.

the remaining three groups doses of the sample. The doses of vitamin A are made up in cocoanut oil and administered orally morning and night for 2 successive days in a volume of 0.1 ml. by means of a tuberculin syringe bearing a 20-gauge needle cut off at 1.5 cm. In the case of low potency products the volume administered can be increased to 0.25 ml. and the dosing time extended to 4 days. Beginning on the day following the last dose vaginal smears are taken daily for 3 successive days and subsequent smears are made according to the type of cells present in the last smear.

A smear is made with pledgets of cotton on a tooth pick, which is moistened with water, introduced into the vagina, rotated once and laid on a slide bearing the number of the rat. A drop of water is added to the slide, the cotton rubbed through this and the smear read without staining under the low power of a microscope. The smears are recorded as indicated in table 2 depending on the predominant type of cells present.

All rats in stages 1 and 2 are regarded as depleted of vitamin A and the remainder as not depleted. As soon as the smears fall into stages 3 and 4, daily smears should be taken and examined so that the end-point of the depletion (stages 1 and 2) may not be missed. The appearance of the smears of the depleted rats is similar to the full estrus smear encountered with ovariectomized rats treated with estrogen or the squamous stage of the normal estrus cycle except a few leucocytes persist in some and stage 2 is included to take care of these.

TABLE 2

Scale used in recording the character of the vaginal smear and the corresponding stage in vitamin A deficiency.

STAGE	SYMBOL	TYPES OF CELLS	STAGE	SYMBOL	TYPES OF CELLS
1	+	Squamous cells	5	+ L — E	Leucocytes with a few epithelial cells
2	± S	Squamous cells with a few leucocytes	6	+ L — S	Leucocytes with a few squamous cells
3	+ S — E	Squamous cells with epithelial cells and a few leucocytes	7	—	Leucocytes
4	+ E — S	Epithelial cells with squamous cells and a few leucocytes			

The response is taken as the number of days, beginning with the first day of dosing, required for the cellular contents of the vagina to change from the squamous cells, stages 1 and 2 (depleted state) to leucocytes or a mixture of leucocytes, epithelial and squamous cells, stages 3 to 7 (curative state) and return to squamous cells, stages 1 or 2 (depleted state). As soon as any rat on a dosage level shows stages 1 or 2 (depleted state) it is returned to the maintenance diet. Three to 4 days after an assay is completed the whole group of rats is weighed, the smears examined and the animals given the vitamin A free diet. When the whole group is uniformly depleted (usually 3 to 5 days later) the rats are weighed again, grouped as above and utilized for another assay. The same group of rats can be used repeatedly in this manner for eight to ten assays or until it becomes too small to use to advantage.

EXPERIMENTAL

To evaluate the method using a relatively wide range of doses and to establish the relation between dose and response which gives the best measure of potency, the following experiment was performed.

Eighty-four rats were depleted of their vitamin A reserves as described above and assembled in six groups of fourteen rats each. Doses of vitamin A from 50 to 524 International Units (I.U.) were prepared from the Canadian Standard Reference oil by dilution with cocoanut oil, each dose being 1.6 times the previous dose. The results are shown in table 3 and graphically in figure 1. It is seen that the points fit a

TABLE 3

The response of rats to vitamin A.

GROUP NO.	NO. OF RATS	DOSE IN I.U.	LOG DOSE IN I.U.	MEAN ARITH. RESPONSE IN DAYS	MEAN LOG RESPONSE IN DAYS	S.E. × 1.96 [1] LOG RESPONSE
1	14	50.0	1.6990	9.00	.9543	.0411
2	14	80.0	1.9031	11.49	1.0602	.0280
3	14	128.0	2.1072	14.94	1.1742	.0269
4	14	204.8	2.3113	18.86	1.2754	.0201
5	14	327.7	2.5155	24.47	1.3888	.0085
6	14	524.3	2.7195	30.47	1.4838	.0109

[1] S. E. = Standard Error = $\sqrt{\frac{\Sigma d^2}{n(n-1)}}$

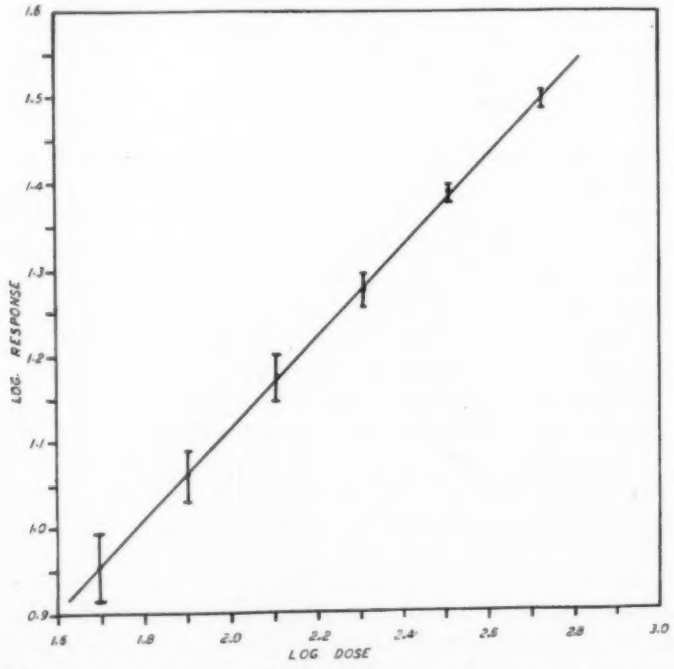

Fig. 1 The logarithmic relation between dose and response to vitamin A. The vertical lines above and below the points are 1.96 × the standard error of the means expressed as logarithms.

straight line reasonably well over this relatively wide range of doses, when the logarithm of the dose is plotted against the logarithm of response. The straight line is represented by the equation log. $y = 1.222 + 0.522$ (log. $x - 2.209$).

The results were subjected to factorial analyses to determine the best method of expressing the values for a linear dosage response curve. Groups 1, 3 and 5 were taken as the standard and 2, 4 and 6 as the sample. Since most graded response assays follow a logarithmic dose and an arithmetic response relationship this was calculated first. According to the variance ratio (table 4, column 2) the dosage response curve shows a significant amount of curvature and the lines are not parallel. The critical value at the 5% level of significance and the number of degrees of freedom in this experiment is approximately 4.0 (Fisher and Yates, '38). On the other hand when the logarithm of the

TABLE 4

The variance ratios using the logarithm of the dose and the arithmetic response and the logarithm of the dose and the logarithm of the response.

	VARIANCE RATIO LOG DOSE — ARITH. RESPONSE	VARIANCE RATIO LOG DOSE — LOG RESPONSE
Sample	114.83	94.16
Slope	1326.09	1140.28
Parallelism	14.38	.18
Curvature	23.21	.07
Opposed curvature	.23	.00

dose and the logarithm of response are used, the variance ratio (table 4, column 3) indicates there is not a significant degree of curvature and the lines are parallel. The variance ratios were calculated in this manner for sixteen assays; and, using the logarithm of the dose and the arithmetic response, the ratio exceeded the critical value in eight cases with respect to curvature and in two cases with respect to parallelism, while using logarithm of the dose and logarithm of response the ratio was well below the critical value in all assays. From these results it is concluded that this type of assay follows a logarithm of the dose and a logarithm of response relationship.

Referring to the data in table 3 using groups 1, 3 and 5 as the standard and 2, 4 and 6 as the sample the most probable value for the ratio M was 1.55 with a range of 1.40 to 1.70 ($p = 0.95$). The actual value was 1.60.

The precision and reproducibility of the method was determined by assaying the Canadian Standard Reference oil against itself. The oil or various dilutions of it were presented as unknowns to the authors. An approximate potency was revealed to permit the preparation of desir-

able dosage levels and the actual potency was reported after the assay and the calculations were completed. Three dosage levels of the standard and unknown in cocoanut oil were used in all these assays and the data were calculated by the method of Bliss and Marks ('39). The assays were set up with equal numbers of rats on each dosage level, but occasional deaths occurred and the whole group for the purpose of the calculations was reduced accordingly by striking off the last animal in the list where extra ones occurred in order to balance the groups. The results are shown in table 5. It is seen that the potency determined by assay agrees satisfactorily with the true potency.

TABLE 5

The precision and reproducibility of the method of assay.

ASSAY	NO. OF RATS	POTENCY FOUND IN	LIMITS OF ERROR OF TEST × 1.96	ACTUAL POTENCY
		I.U.	%	*I.U.*
1	42	1830	87.2 — 114.7	1700
2	54	1730	93.3 — 107.2	1700
3	60	1460	93.7 — 106.6	1445
4	48	780	88.2 — 113.3	790

In order to show the versatile application of the method the results obtained with a group of miscellaneous products are presented in table 6. These products were assayed against the Canadian Standard Reference oil using cocoanut oil as the diluent for the dosage levels except in the case of spinach, mixed vegetable juices, milk chocolate powder and dehydrated egg powder, where water was used. It was necessary to extend the dosing period to 3 days with the butter and the spinach and 4 days with the mixed vegetable juices and egg powder because of their low potency. The dosage levels of these latter products were made adequate by increasing the amounts administered, and the standard in oil was administered in the same manner. The slopes of the dosage response curves of the products in aqueous medium were not significantly different from that of the standard in oil. No difficulties were encountered in these assays and the method appears applicable to a variety of products.

To determine if variations in the initial weight of the rats influenced the precision of the assay, the body weights of all rats in each of four assays were reduced to a common value by analysing the data by covariance (Bliss and Marks, '39). The reduction in the error (table 7) is not sufficient to require a correction or to limit the ranges of body weight below those used in these tests. It is seen from the standard deviation of the mean body weight that considerable variation existed at the time of assembling the groups.

The same sample of cod liver oil was assayed three times against the International Standard β Carotene by (a) the vaginal smear method and (b) by the procedure outlined in the British Pharmacopoeia ('32) addendum ('36) using the 5 weeks dosing schedule. It is seen (table 8) that the vitamin A potency determined by the vaginal smear method is approximately the same as the potency determined by the increase in weight method. The difference between the two weighted means is not significant ($t = 0.526$). The three assays by the vaginal smear method

TABLE 6

Miscellaneous assays.

PRODUCT	NO. OF RATS	POTENCY:	RANGE OF POTENCY: SM × 1.96
		I.U./per gm.	
Cod liver oil	48	1480	1300 to 1680
Shark liver oil	48	27700	24700 to 31000
Distilled vitamin A concentrate	72	307400	286500 to 329800
One per cent vitamin A acetate in Wesson oil	60	46000	41800 to 51300
Dairy butter	55	81	76 to 86
Strained spinach	68	59	52 to 67
Mixed vegetable juices	50	22	17 to 28
Milk chocolate powder	56	184	157 to 215
Dehydrated egg powder	33	7	4 to 11

TABLE 7

The effect of body weight on the variance for experimental error.

ASSAY NO.	NO. OF RATS	MEAN WT. GM.	STANDARD DEVIATION	VARIANCE FOR EXPERIMENTAL ERROR	VARIANCE FOR EXPERIMENTAL ERROR CORRECTED FOR BODY WEIGHT
1	84	136.3	18.3	.00228	.00222
2	48	188.9	16.4	.00257	.00257
3	54	180.5	18.9	.00134	.00129
4	60	171.2	21.1	.00126	.00124

were done on the same group of rats and it required approximately 2 weeks to complete each of them with an interval of 4 to 6 days between assays. On the other hand three groups of rats were required for the increase in weight method which involved a 5-weeks dosing schedule and the assay in each case was prolonged for approximately 7 weeks due to replacements on account of deaths during the first 2 weeks. It is quite evident from these results that the vaginal smear method shows a decided advantage with respect to precision and economy of material, time and effort.

Hickman et al. ('44) have shown that mixed natural tocopherols enhance the growth promoting properties of vitamin A. It was considered advisable to see if tocopherols produced a similar effect in the vaginal smear response to vitamin A. This was done by assaying the Canadian Standard Reference oil against itself and administering α tocopherol [2] or mixed natural tocopherol [3] with the doses at the various levels (total dose) indicated in table 9, assays 1 to 7. Also the International Standard β-Carotene was assayed against itself with the addition of α tocopherol to the sample (assay 8 table 9). It is seen that when α tocopherol is administered with both the standard and the sample in amounts of 0.5 mg., the ratio of the potency of the sample to that of the standard remains unchanged within the limits of error

TABLE 8

A comparison of the vaginal smear method and the increase in weight method for the assay of vitamin A.

ASSAY	NO. OF RATS	POTENCY IN I.U.	LIMITS OF ERROR SM × 1.96 IN PER CENT
		Vaginal smear method	
1	60	1482	88.6 to 112.9
2	56	1561	86.0 to 113.6
3	57	1445	89.9 to 111.2
Weighted mean		1480	93.2 to 107.3
		Increase in weight method	
1	52	1727	82.0 to 124.7
2	48	1165	68.9 to 145.2
3	52	1560	69.4 to 143.7
Weighted mean		1561	84.5 to 118.3

of the assay. There was no evidence from the variance ratios or the slope of the dosage response curve that the tocopherol produced any irregularities in this assay. On the other hand, when α tocopherol or mixed natural tocopherol was added to the doses regarded as the sample, there was a significant difference between the standard and sample in assays 2, 4 and 5. In all these assays the tocopherol shows the power to enhance the activity of the sample, and the mixed natural tocopherol produced a greater effect than the α tocopherol. However, when the α tocopherol was administered, adding 0.5 mg. to the standard and 2.0 mg. to the sample (assay 6), there was not a significant difference between standard and sample. This was repeated (assay 7) with 1.0 mg. of mixed natural tocopherol added to the standard and 4.0 mg.

[2] From the S. M. A. Corporation.

[3] From Distillation Products, Inc.

to the sample with the same result. These experiments indicate that if an oil is suspected of containing tocopherol the true vitamin A activity can be determined if an amount of tocopherol above the optimum (0.5 mg.) is administered with the standard and a similar amount added to the doses of the sample since an excess above 0.5 mg. does not produce an added effect. The experiment with β carotene illustrates that tocopherol enhances its activity since there is a significant difference between

TABLE 9

The effect of tocopherols on the response to vitamin A.

ASSAY NO.	NO. OF RATS	RATIO OF SAMPLE TO STANDARD	LIMITS OF ERROR IN PER CENT SM × 1.96	VARIANCE RATIO FOR SAMPLES	REMARKS
1	54	1.02	93.5 to 107.1	0.276	Canadian Standard Reference oil with 0.5 mg. α tocopherol added to both standard and sample.
2	44	1.22	88.7 to 112.7	13.04	0.5 mg. α tocopherol added to sample.
3	28	1.20	79.0 to 126.5	2.83	0.5 mg. α tocopherol added to sample.
4	44	1.56	79.9 to 125.1	17.10	4.0 mg. mixed natural tocopherol added to sample.
5	44	1.62	77.0 to 129.8	19.40	1.0 mg. mixed natural tocopherol added to sample.
6	60	0.96	86.7 to 115.3	0.41	0.5 mg. α tocopherol added to standard and 2.0 mg. α tocopherol to sample.
7	44	1.01	88.0 to 113.6	0.11	1.0 mg. mixed natural tocopherol added to standard and 4.0 mg. mixed natural tocopherol to sample.
8	72	1.10	93.4 to 107.4	4.11	β carotene with 0.5 mg. α tocopherol added to the sample.

the standard and sample according to the variance ratio. These experiments confirm those of Hickman et al. ('44) indicating that tocopherols augment the effect of vitamin A, but this augmentation can be controlled in an assay by ensuring that tocopherol is administered with the doses in amounts above the optimum to both standard and sample.

It was observed that the squamous cells in the vaginal smear of depleted rats disappeared 3 to 5 days after dosing with vitamin A. Attempts were made to use the absence of squamous cells from the smears as a criterion of response in the same manner as the absence of leucocytes is used as a criterion of response in the assays of estrogens. Rats

were depleted as described above and assembled for four dosage levels of vitamin A. The doses were administered orally once daily for 5 days and the smears were examined daily for 6 or more days after dosing. The percentage of rats on each dosage level showing the absence of squamous cells in their smears between the third and fifth day after dosing was taken and the slope of the dosage response curve calculated according to the method of Bliss ('35) for the quantal response data. It is seen (table 10) that the slope (b) of the dosage response curve is relatively low for a precise method of assay. Difficulties were encountered in obtaining clear-cut end points and this criterion of response was rejected in favor of the one described above.

TABLE 10

Quantal response assays for vitamin A.

CURVE NO.	NO. OF RATS	b	vb	RD_{50} I.U.
1	46	5.045	1.943	99.7
2	60	3.095	2.704	129.2
3	57	4.325	1.281	129.2
4	61	1.445	0.987	95.4
5	34	3.146	3.508	109.2
6	65	1.568	0.216	136.6
Mean		2.103		

DISCUSSION

The assay of vitamin A by its effect on growth rate, as measured in terms of the weight of rats fed on a relatively purified diet deficient in vitamin A and undoubtedly in other factors as well, makes such an assay method unsatisfactory on account of the relatively non-specific type of response. While vitamin A has some effect on growth there are a number of factors concerned in such a response, and vitamin A is not necessarily the limiting one under the existing conditions. On the other hand the change produced in epithelial structures is one of the most characteristic effects of a vitamin A deficiency. It is completely reversible and occurs regardless of age. The role of vitamin A in the normal differentiation of cells has thus far not been explained satisfactorily. The production of keratinized cells in the cellular contents of the vagina of rats deficient in vitamin A makes a readily available and practical means of measuring the response to vitamin A.

The diet usually employed to produce a vitamin A deficiency is not well balanced with respect to the B vitamins; and to render the rats deficient in vitamin A but in an otherwise more satisfactory nutritional condition, a maintenance diet was employed for the primary depletion

period and to support the rats at low levels of vitamin A reserve between tests. A number of substitutions and additions to the A free ration were tried in an attempt to devise a satisfactory maintenance diet, namely (a) feeding the vitamin A free diet and a maintenance dose of vitamin A, (b) adding skim milk powder to the amount of 3% of the diet, (c) substituting unextracted commercial casein for extracted casein in the A free diet, (d) adding wheat germ to the A free diet to the amount of 5, 7 and 10%, and (e) adding wheat germ to the amount of 7% and substituting unextracted casein for extracted casein and brewers' yeast for fat-free yeast. The last alteration of the A free diet proved more satisfactory and convenient. When the maintenance diet (table 1) was prepared at weekly intervals in this manner and fed during the primary depletion period, a steady increase in weight occurred and the rats were in a better nutritional condition than when the A free diet alone was fed. The amount of carotene in the maintenance diet appears to be just enough for subsistence and at the same time to render the rats easily depleted upon feeding the A free diet. No difficulties have been encountered with different lots of commercial casein and wheat germ obtained over a period of 2 years.

Attempts were made to hasten vaginal introitus by the administration of estrogens. Following subcutaneous injection of an aqueous solution of diethylstilbestrol (0.2 ml. = 2 μg.) into a group of 30 rats 25 to 35 days of age and estrone (0.2 ml. = 5 μg.) into another group of 30 rats of the same age, rupture of the vaginal membrane occurred 4 to 6 days later and the rats were ready for ovariectomy. Although this treatment is unnecessary it has the advantage of saving time in checking the rats for vaginal introitus during the primary depletion period. In a few cases when puberty was allowed to proceed naturally, and vaginal introitus was delayed in a few rats in the group, the membrane over the vagina was punctured with a needle. This intervention and the administration of estrogens did not interfere with the subsequent reaction of the rats to vitamin A.

Intact female rats have been used by other workers to demonstrate the effect of vitamin A on the cellular contents of the vagina. It was considered that the ovariectomized rat would be a more satisfactory animal to use, since the removal of the ovaries ensures that the response will not be interpreted as a normal or a prolonged estrus effect. No post-operative infections occurred when the vitamin A deficient rats were ovariectomized without precautions as to sterility, and no instances of regeneration of ovarian tissue have been encountered. This operation is a relatively simple one requiring 3 to 4 minutes per rat,

and after one has obtained experience in administering the anaesthetic the deaths at operation are nil.

The progress of the depletion and the response is quite adequately followed by the system of symbols recommended. In some cases the smears are slightly difficult to interpret due to the persistence of leucocytes. This usually occurs when the rats are suffering from an infection of the urogenital or respiratory tracts. A confirmatory reading often clears up any doubts regarding the end point of the test. It was found that as soon as the cells in the smears become predominately squamous in the vitamin A depleted rats, such a condition persists until death occurs. The smears are examined only once daily, since it was considered impractical and inconvenient to read them more frequently. Smears may be read quite satisfactorily 24 hours after they are obtained providing they are moistened and covered. This practice of having an assistant take the smears on Sunday and to delay the reading until Monday was followed in a few assays.

The response of the rats on the low dosage level was questionable in a few assays because the dose of vitamin A was not sufficient to cause a change from the squamous cells to epithelial cells and leucocytes during the 3 days after dosing. Groups of rats reacting in this manner were excluded from the calculations. It has been observed that the dosage levels can be arranged for unknown oils quite satisfactorily when the L value per gram as determined colorimetrically by the method of Dann and Evelyn ('38) is interpreted directly in International Units. The minimum total dose for a satisfactory response in our colony of rats is between 45 and 60 I.U. of vitamin A.

Greater uniformity is obtained if complete groups of rats are assembled for a test, rather than to begin dosing a few rats at a time as they become depleted. The former method requires some shifting of the rats from the A free diet to the maintenance diet and vice versa, but it is only in the primary depletion period that this shifting requires much attention. After the first assay is completed the rats become depleted at a remarkably uniform rate for subsequent assays.

A linear dosage response relationship is obtained by plotting the logarithm of the dose against the logarithm of the response and in this respect the method differs from most assay procedures of a continuous variate type in which a linear relationship is obtained by plotting the logarithm of the dose against the arithmetic response. In this connection De Graff et al. ('41) have shown that the assay of digitalis glucosides by the embryonic chick heart follows a logarithm of the dose and a logarithm of the response relationship. To show that the varia-

tions in response of the individual dosage levels follow a normal distribution curve, the response obtained with each of three dosage levels of the Canadian Standard Reference oil were pooled for eleven assays which gave 100 rats per dosage level. When the days were grouped for each dosage level and the resulting percentages calculated, a normal curve of distribution for each dosage level was obtained.

The precision of this method of assay is relatively greater than that obtained with the increase in weight method of assay. Typical values of the limits of error attainable by the method using different numbers of rats per assay are shown in tables 5 and 6 and a direct comparison with the increase in weight method is shown in table 8. From these results an error of $\pm$ 12 to 15% using 60 rats per assay and p at 0.95 is quite readily attained. Most of the assays reported here have been calculated by the method of variance and covariance of Bliss and Marks ('39) to show that they fulfill the requisites of a satisfactory method of biological assay. This method of calculation requires the dosage levels to be adjusted at equally spaced intervals and that equal numbers of rats be used for each level. These requirements are not readily obtainable in routine assays due to deaths of a few rats from respiratory infections during the course of an assay and the adjustment of the dosage level of low potency products. The method of calculating biological assays of a continuous variate type proposed by Irwin ('37), in which a system of weighting for numbers of rats on each dosage level is used, was employed for a few assays that did not meet the above requirements and in order to check the results obtained by the method of Bliss and Marks ('39).

The assays reported in table 6 show the method to be applicable to a variety of products. To attain desirable dosage levels with some of the products it was necessary to continue the dosing period for more than 2 days. There was no evidence that this alteration in technique produced results different from the dosing period of 2 days recommended for fish oils. The slopes of the dosage response curves obtained for products in aqueous medium were not significantly different from the slopes obtained for the standard in an oily vehicle.

The reduction in the error of the method obtained by correcting for body weight does not appear to be significant from the results obtained in the assays in table 7. In all the assays conducted the rats have been assembled on the basis of equal distribution of body weight in each group, and variations in weight within the groups on an assay have been as much as 50 gm. It appears from the results obtained that the response is independent of body weight within these limits. Although

considerable uniformity in depletion time during the primary depletion has been observed to occur within litters, no attempt has been made to pair off litter mates in the assays due to the impracticability of such a procedure.

The observation by Hickman et al. ('44) that vitamin E augments the activity of vitamin A has been confirmed. Most of the evidence favors the conclusion that the tocopherols act as anti-oxidants during the absorption of vitamin A from the gastro-intestinal tract. Hickman et al. ('44) have shown that the maximum effect is produced at approximately 0.5 mg. of tocopherol per rat per day. On this basis, a product containing vitamin A as well as vitamin E can be assayed quite satisfactorily by administering this amount of tocopherol with the dosage levels of vitamin A to both standard and sample. Tocopherol itself was shown to possess no vitamin A activity when administered to a group of 20 rats in amounts of 340 mg. per rat. It does not appear practicable to prevent errors arising from the tocopherol effect by the addition of tocopherol to the diet because it is readily oxidized in such a dietary medium; also the addition of a natural product containing vitamin E does not appear feasible because most of them contain significant amounts of carotene. Since tocopherols in products assayed for vitamin A yield an added biological effect, errors can quite conceivably occur in the determination of vitamin A activity by the spectrophotometric or chemical methods when the results are reported in terms of biological activity. Considerable evidence is available indicating that such methods do not give reliable estimations of the biological activity of the product due to the different forms in which vitamin A may exist, hence the necessity of a precise biological assay method for vitamin A.

It was disappointing to obtain the relatively low values for the slopes of the dosage response curves with the accompanying low degree of precision in the quantal response method of assay shown in table 10. This method had the advantage of being short, and convenient. Preliminary assays using other dosing times were tried without any success in improving the precision of the method.

SUMMARY

A method of biological assay of vitamin A and its precursors based on the changes produced in the cellular contents of the vagina of ovariectomized rats is described. The dosage response relationship was analysed by methods of variance and covariance and shown to be logarithmic. The method shows considerable advantage over the increase in weight method with respect to precision and economy of time,

material and effort. The enhancement of the biological activity of vitamin A by tocopherols has been confirmed and a method of overcoming this effect in the estimation of vitamin A activity is described.

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The American Institute of Nutrition will make this award in recognition of distinctive research by investigators in the United States and Canada which has emphasized the nutritive significance of the components of milk or of dairy products. The award will be made primarily for the publication of specific papers, but the judges may recommend that it be given for important contributions over an extended period of time. The award may be divided between two or more investigators. Employees of the Borden Company are not eligible for this honor.

The formal presentation will be made at the annual meeting of the Institute at Cleveland, May 8, 1945. To be considered for the award, nominations must be in the hands of the Chairman of the Nominating Committee by January 15, 1945. The nominations should be accompanied by such data relative to the nominee and his research as will facilitate consideration for the award.

FREDERICK J. STARE
Harvard Medical School
Boston, Massachusetts

CHAIRMAN, NOMINATING COMMITTEE

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Nominations are solicited for the 1945 Award of $1,000 established by Mead Johnson and Company to promote researches dealing with the B complex vitamins. The recipient of this Award will be chosen by a Committee of Judges of the American Institute of Nutrition and the formal presentation will be made at the annual meeting of the Institute at Cleveland on May 8, 1945.

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To be considered by the Committee of Judges, nominations for this award for work published in 1944 must be in the hands of the Secretary by January 15th, 1945. The nominations should be accompanied by such data relative to the nominee and his research as will facilitate the task of the Committee of Judges in its consideration of the nomination.

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SECRETARY, AMERICAN INSTITUTE OF NUTRITION